Universidade de Aveiro Departamento de Biologia
2015

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Efeito de substratos no secretoma e subproteoma de células estaminais mesenquimais

Substrates effect on Mesenchymal Stem Cells (MSCs) secretome and cellular subproteome

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Mário Grãos, Investigador Auxiliar do Biocant e CNC - Centro de Neurociências e Biologia Celular.

o júri

presidente

Professora Doutora Maria Helena Abreu Silva
Professora auxiliar, Universidade de Aveiro

Doutor Bruno José Fernandes Oliveira Manadas
Investigador Auxiliar, CNC-Universidade de Coimbra

Doutor Mário Martins Rodrigues Grãos
Investigador Auxiliar, Biocant e CNC - Centro de Neurociências e Biologia Celular

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palavras-chave

Células estaminais mesenquimais; Mecanotransdução; Rigidez; Secretoma, subproteoma

resumo

As células estaminais mesenquimais (MSCs) são células estaminais adultas, multipotentes, capazes de se auto-renovar e diferenciar em diferentes tipos celulares mesodermais. O isolamento destas células pode ser feito a partir tecidos mesenquimais, bem como de extra embrionários, como por exemplo da matriz do cordão umbilical. Este último constitui uma fonte alternativa e atrativa de MSCs uma vez que estas apresentam características mais *naïve* e uma maior taxa de proliferação.

In vivo, as células mesenquimais têm um microambiente especializado que é essencial à regulação da sinalização, proliferação, migração e diferenciação celular. Através da mecanotransdução, as forças mecânicas do ambiente extracelular são traduzidas em respostas bioquímicas que podem conduzir à alteração de fenótipo e diferenciação em diferentes tipos celulares e, além disso, à alteração da síntese de proteínas.

A partir destas observações, este trabalho teve como objetivo o estudo do efeito das propriedades do substrato, tais como a rigidez e composição bioquímica, no secretoma e subproteomas de células estaminais mesenquimais isoladas a partir da matriz do cordão umbilical. Este estudo revelou uma modulação do secretoma e subproteomas celulares (frações solúvel e membranar) quando as MSCs foram mantidas em cultura sobre substratos com menor rigidez, demonstrando a modulação de múltiplas proteínas e nomeadamente o aumento dos níveis de proteínas anti-oxidantes. Deste modo, propomos que células cultivadas em substratos com menor rigidez possuam uma maior atividade anti-oxidante, o que irá permitir que estas apresentem uma melhor resposta face a ambientes hostis *in vivo* num contexto de transplantação.

keywords

Mesenchymal stem cells, Mechanotransduction, Stiffness, Secretome, subproteome

abstract

Mesenchymal stem cells (MSCs) are adult multipotent cells that possess self-renewal capacity, have a high proliferative ability and are able to differentiate into mesodermal cell types. MSCs can be isolated from mesenchymal and extraembryonic tissues such as the umbilical cord matrix. The latter constitutes an attractive and alternative source of MSCs since these cells seem to be more naïve and possess higher proliferation capacity.

In vivo, MSCs reside in a specialized microenvironment that is essential for the regulation of signaling, proliferation, migration and differentiation. Through mechanotransduction, mechanical forces of microenvironment are transduced into biochemical responses, which can lead to alterations in phenotype and lineage-specific differentiation, and changes in protein synthesis of MSCs. Based on these observations, this study aimed at exploring the effect of physical and biochemical substrate composition on the secretome and cellular subproteomes of MSCs derived from umbilical cord matrix. The present study revealed the modulation of the secretome and cellular subproteome profiles (soluble and membrane fractions) of MSCs cultured on soft substrates, with several proteins being modulated, namely the up-regulation of antioxidant proteins. Hence, we propose that MSCs cultured on soft substrates may constitute a population of cells with increased antioxidant properties, in principle allowing the cells to cope better with the stressful and hostile environments that they may encounter *in vivo* in a transplantation context.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS:

A	ACN	Acetonitrile
	Alpha-MEM	Alpha modified Eagle's medium
	3-APTMS	3-Aminopropyltrimethoxisilane
B	BM	Bone Marrow
	BM-MSCs	Bone marrow mesenchymal stem cells
C	CD	Cluster of differentiation
	CFU-F	Colony forming unit-fibroblast capacity
	CV	Coefficient of variation
D	DMSO	Dimethyl sulfoxide
E	*E*	Elastic or Youg's modulus
	EDTA	Ethylenediamine tetraacetic acid
	EGFP	Enhanced green-fluorescent protein
	ECM	Extracellular matrix
	ESCs	Embryonic stem cells
F	FA	Formic acid
	FAK	focal adhesion kinase
	FAs	Focal adhesions
	FBS	Fetal bovine serum
	FGF-2	Fibroblast growth factor-2
	FN	Fibronectin
G	*G*	Shear modulus
	GM-CSF	Granulocyte-macrophage colony-stimulating factor
	GO	Gene ontology

	GOrilla	Gene Ontology enRIchment anaLysis and visuaLizAtion
	GT	Generation time
H	HLA	Human leukocyte antigens
I	IDA	Information-dependent acquisition
	IFN-γ	Interferon gamma
	IL	Interleukine
L	LC	Liquid chromatography
	LINC	Linker of Nucleoskeleton to Cytoskeleton
M	MS	Mass spectrometry
	MHC	Major histocompatibility complex
	MSCs	Mesenchymal stem cells
O	Oct4	Octamer-binding transcription factor 4
P	PANTHER	Protein ANalysis THrough Evolutionary Relationships
	PBS	Phosphate buffered saline
	PD	Population doubling
	PDMS	Polydimethylsiloxane
R	RhoA	Ras homolog gene family, member A
	RT	Room temperature
S	SD	Standard deviation
	SEM	Standard error of the mean
	Sox2	SRY (sex determining region Y)-box 2
	SPE	Solid phase extraction
	SWATH	Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra

T	TC	Tissue culture
	TCA	Trichloroacetic acid
	TCPS	Tissue culture polystyrene
	TGF-β	Transforming growth factor beta
	TNC	Total number of cells
	TNF-α	Tumor necrosis factor alpha
	TOF	Time of flight
U	UC	Umbilical cord
	UCB	Umbilical cord blood
	UCM	Umbilical cord matrix
V	VEGF	Vascular endothelial growth factor
W	WJ	Wharton's jelly
	WJ-MSCs	Wharton's jelly mesenchymal stem cells

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. MESENCHYMAL STEM CELLS (MSCS)

Mesenchymal stem cells (MSCs), also classified as mesenchymal stromal cells or mesenchymal progenitor cells, were first characterized by Friedenstein and colleagues[1] as an adherent and fibroblast-like population of cells from the stromal compartment of bone marrow (BM). MSCs are multipotent adult stem cells known by their unique characteristics, such as their undifferentiated state and ability to self-renew, high proliferative capacity, formation of clonal cell populations due to their colony forming unit-fibroblast (CFU-F) capacity, and multilineage differentiation potential[1–3]. These cells can differentiate into mesoderm-type cells like osteocytes, chondrocytes and adipocytes and this capacity is crucial to define *bona-fide* MSCs[2,4] . Recent studies report that MSCs are also capable to differentiate *in vitro* into non-mesenchymal lineages, such as neural-like lineages[5].

1.2. SOURCES OF MESENCHYMAL STEM CELLS

Bone marrow (BM) represents the primary and the most common source of MSCs[1]. However, this source involves an invasive isolation and the proliferative capacity and potential of differentiation decrease with donor's age[6,7], hence, alternative sources for isolating MSCs have been explored.

Mesenchymal stem cells can be isolated from several adult tissues such as skeletal muscle, adipose tissue[8], dental pulp and other dental tissues[9].

In addition, MSCs can be found in extra-embryonic tissues like the umbilical cord (UC) and umbilical cord blood (UCB), placenta and amniotic fluid[10,11]. Extra-embryonic tissues are a good alternative source of MSCs since these appear to be more naïve and possess higher multipotent plasticity, have lower immunogenicity[12,13] and faster proliferation[14] than MSCs from adult mesenchymal tissues. Besides that, extra-embryonic tissues have an early stage of development and possibly less incorporated mutations when compared with adult tissues[15].

1.2.1. UMBILICAL CORD MATRIX – *WHARTON'S JELLY (WJ)*

The umbilical cord (UC) is an extra-embryonic formation that contains two arteries, a vein and connective tissue that surrounds the vessels, named *Wharton's jelly* (WJ)[16]. *Wharton's jelly* is the major component of extracellular matrix of umbilical cord and a rich source of MSCs. It is largely composed by collagen and proteoglycans[5,12] providing a natural microenvironment for MSCs, and has stromal support properties[17]. Within the *Wharton's jelly*, MSCs can be isolated from three different regions — perivascular, intervascular and subamnion —, representing different sub-populations of MSCs. Besides UC tissue, umbilical cord blood (UCB) also contains MSCs[18], although in much lower amounts. The currently existing techniques for harvesting and expanding UCB-MSCs are not robust enough to counterbalance the low frequency of these cells[17]. All compartments of UC where MSCs can be found are represented in Figure 1.

Since umbilical cord is considered medical waste, it is a non-controversial source and does not raise ethical problems for its collection. Furthermore, UC allows the isolation of a large initial number of cells, avoiding extensive proliferation and eventual genetic and epigenetic damage[17] and cells are collected through a simple, non-invasive and painless process[19].

UC-MSCs share many properties with adult BM-MSC, including most of the typical MSCs immunophenotypic markers in common with BM-MSCs (Table 1).

In addition, WJ's-MSCs show higher proliferative capacity and shorter doubling population time, have higher CFU-F frequency and greater *ex vivo* expansion capacity[5]. WJ's-MSCs are reported to possess some characteristics similar to embryonic stem cells (ESCs), including the potential of differentiate into cell types of the three germ layers[3], the expression of stem cell markers such as Oct-4, Sox-2, and Nanog[7,20], with the advantage that MSCs do not form teratomas upon transplantation[17]. Nevertheless, these similarities with ESCs are still controversial and the focus of ongoing research. However, UC-MSCs are considered a more primitive population.

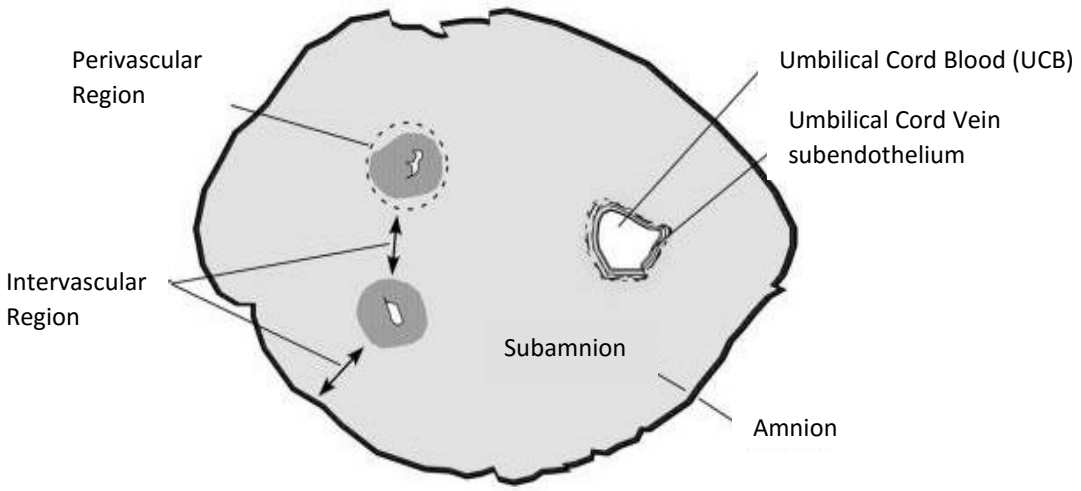

Figure 1. Human Umbilical Cord (UC) section with distinct regions that contains mesenchymal stem cells. Umbilical Cord Blood (UCB); *Wharton's Jelly* (includes Subamnion, Intervascular and Perivascular regions); and Umbilical vein subendothelium. Adapted from Troyer and Weiss, 2008[17].

WJ-MSCs are CD73, CD90, CD105 and HLA class I positive, and CD45, CD34 and HLA class II negative[10,21]. These cells are also plastic-adherent and have fibroblast-like morphology when cultured *in vitro*. WJ-MSCs are multipotent (adipo-, chondro- and osteogenic potential), hence sharing all minimal criteria used to define MSCs as proposed by the Internal Society for Cellular Therapy[22,23]. Moreover, according to the literature, MSCs from WJ showed a higher therapeutic potential compared with other sources due to their immune-privileged nature, which could mean a reduced incidence of graft-versus-host disease, in transplantations[17]. WJ's-MSCs also have higher proliferation rates, better immunomodulatory properties and longer maintenance of multipotent characteristics during time, *in vitro*[22].

Table 1. Surface marker expression of BM-MSCs and UC-MSCs (adapted from Anzalone et al., 2010[10]).

Markers	*BM-MSC*	*WJC*
CD10	+	+
CD13	+	+
CD14	−	−
CD29	+	+
CD31	−	−
CD33	−	−
CD34	−	−
CD44	+	+
CD45	−	−
CD49e	+	+
CD51	+	+
CD54	+	NA
CD56	+	−
CD59	+	NA
CD68	NA	+
CD71	+	NA
CD73	+	+
CD79	−	NA
CD80	−	+
CD86	−	−
CD90	+	+
CD105	+	+
CD117	−	+
CD163	NA	−
CD166	+	+
CD235a	−	NA
CK-7	NA	−
CK-8	NA	+
CK-18	+	+
CK-19	+	+
Connexin-43	+	+
GATA-4	+	+
GATA-5	NA	+
GATA-6	NA	+
GFAP	+	+
HLA-A	+	+
HLA-B	+	+
HLA-C	+	+
HLA-DR	−	−
HLA-G	+	+
HNF-4α	NA	+
Nanog	+	+
Nestin	+	+
NSE	+	+
Oct3/4A	+	+
α-SMA	+	+
Vimentin	+	+

Abbreviation: NA, not applicable.

1.3. MECHANOTRANSDUCTION

In vivo, cells reside in a specialized microenvironment that is essential for the regulation of signaling, proliferation, migration and differentiation[24,25]. This microenvironment consists of soluble and surface-bound signaling factors, cell-cell contacts and the extracellular matrix (ECM), which is its major component[25].

Through cell-ECM adhesion sites (multimeric protein complexes called Focal Adhesions — FAs), cells can sense and integrate (physiological) mechanical stimuli and convert them into intracellular signaling through mechanotransduction mechanisms. Hence, cells can sense and respond to complex biochemical and biophysical signals that will influence the cell's behavior, morphology, dynamics, and fate[25,26]. Mechanotransduction has been showed to modulate lineage specification of MSCs by integrating the biochemical and biophysical properties of the ECM, triggering signal transduction cascades[26], contributing to cell fate decisions in addition to other stimuli presented to cells, such as those provided by soluble factors. This modulation includes short-term responses, such as alterations in intracellular tension, adhesion, spreading or migration; as well as changes in long-term effects, such as protein synthesis and secretion, structural reorganization, proliferation and viability. In addition to FAs, a variety of molecules and subcellular structures have been shown to mediate force-sensing and mechanosignaling, such as primary cilia, cell-cell adhesions (such as Adherens Junctions), stretch-modulated ion channels, the nucleus and the cytoskeleton[27] **(Figure 2).**

1.3.1. MECHANISMS OF MECHANOTRANSDUCTION

The direct involvement of ECM in signal transduction through integrin receptors has been studied in order to understand how matrix elasticity influences cell behavior. Integrins mediate cell adhesion to the ECM and this creates a dynamic relationship whereby the ECM can transduce signals into the intracellular environment or, on the other hand, signaling from the cell can also change the ECM. Thus, integrin-mediated cell-ECM adhesions (Focal Adhesions) act as a bridge between the extracellular environment and the cellular cytoskeleton that contains the primary regulator of cellular traction forces — actin-myosin based contractility[28].

As introduced above, it is thought that cells sense and respond to biophysical signals through integrin-mediated focal adhesion (FA) signaling **(Figure 2).** Focal adhesions are cell membrane regions involved in cellular attachment to ECM proteins (such as fibronectin, collagen or laminin) through transmembrane proteins named integrins. Thus, mechanotransduction initiates at focal adhesions by inducing local conformational changes, followed by signal transduction cascades inside the cytoplasm. Intracellular domains of integrins interact with adapter and signaling molecules such as talin, paxilin, vinculin and α-actinin allowing the direct physical linkage to the actin cytoskeleton[25,27,29].

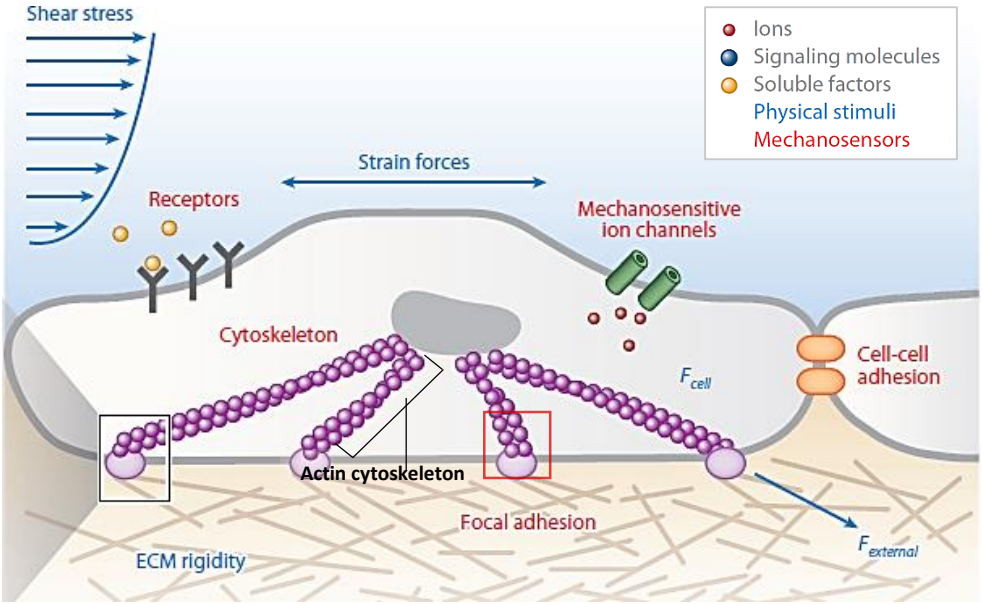

Figure 2. Biophysical signals and molecular interactions between cells and their microenvironment. Biophysical signals in cells niche include extracellular matrix (ECM) rigidity and topography, flow shear stress, strain forces, and other mechanical forces exerted by adjacent support cells. $F_{external}$ and F_{cell} shows the balance of external and internal forces, respectively. Stem cells can sense these stimuli through mechanosensors such as ion channels, focal adhesions, cell surface receptors, actin cytoskeleton, and cell-cell adhesions, (Adapted from Sun et al., 2012[25]).

In response to mechanical stimuli, many enzymes change their kinetics, namely focal adhesion kinase (FAK) that once activate induces several signaling pathways[30]. This activation may regulate diverse cell functions such as migration, differentiation and proliferation. Additionally, FAK can also activate RhoA that is a key regulator of actin cytoskeleton tension. Active RhoA recruits myosin II to bind actin cytoskeleton, leading to an increase of cytoskeleton tension and FAs reinforcement by recruitment of further FA proteins. Sequentially, actomyosin cytoskeleton contracts which results in a retrograde movement of actin fibers. The stiffer the extracellular environment, the higher the tension transmitted to the nucleus through the LINC complex, which constitutes a protein complex physical bridge between the cytoskeleton and the nuclear lamina[24,29,31]. Hence, mechanical signals are transmitted to the nucleus and modulate the nuclear shape, — as well as the shape of the whole cell —, which may vary depending on tension forces sensed and exerted by cells. With increased tensile forces cells present a more elongated nucleus in contrast to a more round shape when low forces are acting[31]. Generally, more tension forces are related to increased stiffness of substrates, while low tension forces are related

to soft ones. This phenomenon reflects the reaction of cells to their environment, constantly probing the environment and responding according to the biophysical conditions that are present.

1.3.2. STIFFNESS

Several cell types, and in particular stem cells (including MSCs), are sensitive to ECM or substrate mechanics (when *in vivo* or *in vitro,* respectively), where distinct degrees of stiffness can drive their differentiation into specific lineages or, for instance, maintain stem cells in a pluripotent or multipotent state. Although the direct activation of signal transduction through integrin receptors by extracellular matrix proteins has been well studied[32], the mechanisms through which physical properties of the ECM — such as stiffness — are transduced into the cells are still not fully understood.

The stiffness of a material is measured by the ratio between applied forces and the resulting stretch. In a biological context, a material stiffness or elasticity is referred to as Young's or Elastic modulus (**E**) and can be defined as ratio of force per unit area needed to deform a material by a given fractional amount without any permanent deformation. So, a high elastic modulus corresponds to high stiffness and low deformability[36]. To quantify the rigidity of a material, the Elastic modulus (**E**) and shear modulus (**G**) are commonly used. For the determination of the elastic modulus (**E**), the force is applied perpendicularly to the material's surface, while for shear modulus the applied force is parallel to the surface (**Figure 3**). The function $E=2G(1+\nu)$, where ν is the Poisson ration, relates elastic and shear modulus. In cases when materials do not change volume under stretch, the Poisson ratio is 0.5 and consequently the elastic modulus will be three times its shear modulus. The units for rigidity are force per unit area, the SI unit being Pascal (Pa), which in a biological context can be commonly represented as $nN/\mu m^2$ or kPa[30].

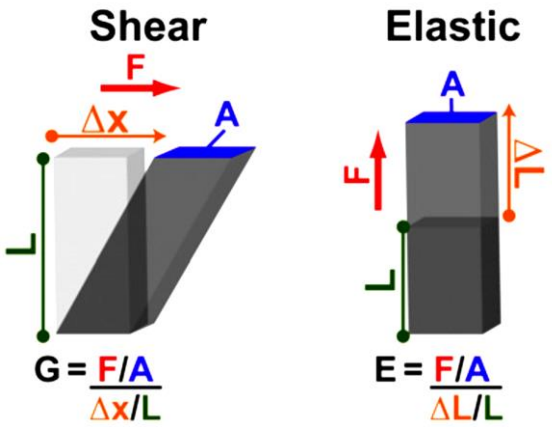

Figure 3. Shear and Elatic Moduli. Shear *(G)* and Elastic moduli *(E)* are the ratio of **stress** over **strain.** While **stress** is the amount of force applied per area (F/A), **strain** represents the displacement in the direction of the applied force relative to initial length (Δx/ΔL). The direction of the applied force differs on shear and elastic modulus. While for the elastic modulus (***E***), the force is applied perpendicularly to the material's surface, for shear modulus the applied force is parallel to the surface. (Adapted from Moore et al., 2010[30]).

Tissues in the body exhibit different physical properties, namely stiffness. The elastic moduli of mammalian tissues (not being static) range from 0.1 nN/μm^2 to 30,000,000 nN/μm^2 (**Table2**). It may vary with age, as it is the case of brain (by decreasing), or during a pathology, including muscular dystrophies, cardiomyopathies or cancer progression and metastasis[30,31].

Table 2. Elastic modulus of several mammalian tissues. (Adapted from Moore et al., 2010[30]).

Tissue Type	Elastic Modulus (nN/μm^2)
Brain	0.1-10
Muscle	12-100
Fat	20
Artery	100-3,800
Areolar connective tissue (fibroblasts in collagen)	600-1,000
Bone	17,100,000-28,900,000

Usually cells reflect a more physiological-like behavior *in vitro* when cultured on materials with stiffness similar to their native microenvironment[30]. According to Engler et al., 2006 [34],matrix elasticity has profound effects on the differentiation of mesenchymal stem cells. Soft matrices promote

differentiation into neuronal-like cells, moderate elasticity favor myogenic differentiation and rigid matrices leads cells to osteogenic differentiation [32,34] (Figure 4).

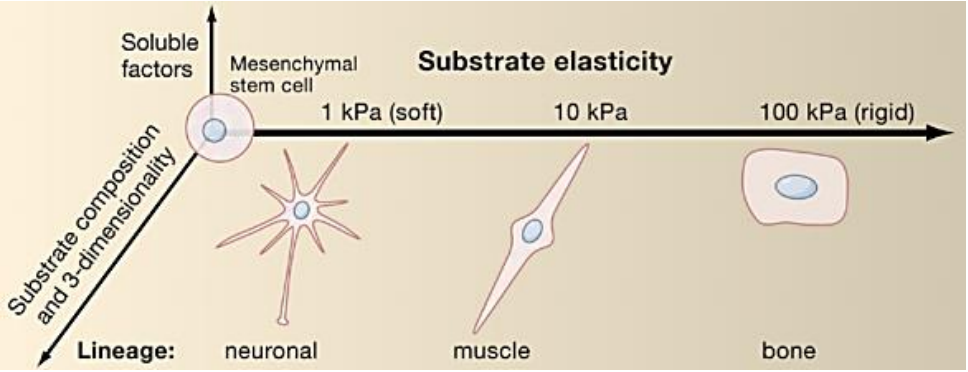

Figure 4. Multiple factors regulating stem cell fate. Factors like secreted soluble factors; elasticity, biochemical composition and dimensionally of substrate have profound effect on mesenchymal stem cell differentiation. According to the literature, soft matrices favored neuronal-like cell differentiation, moderate elasticity promoted myogenic differentiation and rigid substrates stimulated osteogenic differentiation (Adapted from Even-Ram,S. et al., 2006[32]).

Assuming that cells are sensitive to biophysical signals encoded within the ECM, biomaterials can be used as a model to assess the effect of changing biochemical composition, topography or physical environment on cell behavior. One of the mechanical cues that can be investigated (by mimicking the ECM) is stiffness, which has been shown to have influence on cell proliferation, motility, stem cell differentiation and other cell functions. Nonetheless, stiffness responses depend on both the nature of the matrix and the cell-type specific components involved in the responses.

1.4. PROTEOME AND SECRETOME OF MSCS

The dynamic contact of cells with their niche has direct impact on cell phenotype and is a key regulator during proliferation and differentiation. The secreted molecules by MSCs are involved in intercellular communication and participate in many physiological processes, such as cell adhesion, binding and signaling, differentiation, invasion, metastasis, angiogenesis, and apoptosis; and contribute to the maintenance of homeostasis at the level of organs, systems or even the whole organism. This complex set of secreted molecules is defined as Mesenchymal stem cell secretome[35,36].

The therapeutic potential of mesenchymal stem cells can be attributed to the fact that (i) these cells ''home'' or migrate to specific areas of acute injury via chemical gradients, (ii) act by local restoration

of function by differentiating into multiple cell types to augment or replace damaged tissues and (iii) therapeutic potential can be attributed to the secretion of bioactive factors with potential for affecting both local and systemic physiologic processes and tissue repair. Considering the latter, MSCs exert a therapeutic effect via secretion of bioactive factors with antiapoptotic, antiscarring and neovascularization effects, as well as immunomodulatory properties[35].

Park et al., 2007[36] provided a comprehensive compilation of human MSCs' proteome. Some information such as extracellular matrix proteins and cytokines, chemokines and growth factors of MSCs[36] is summarized in **Figure 5**, which is in agreement with other studies[37].

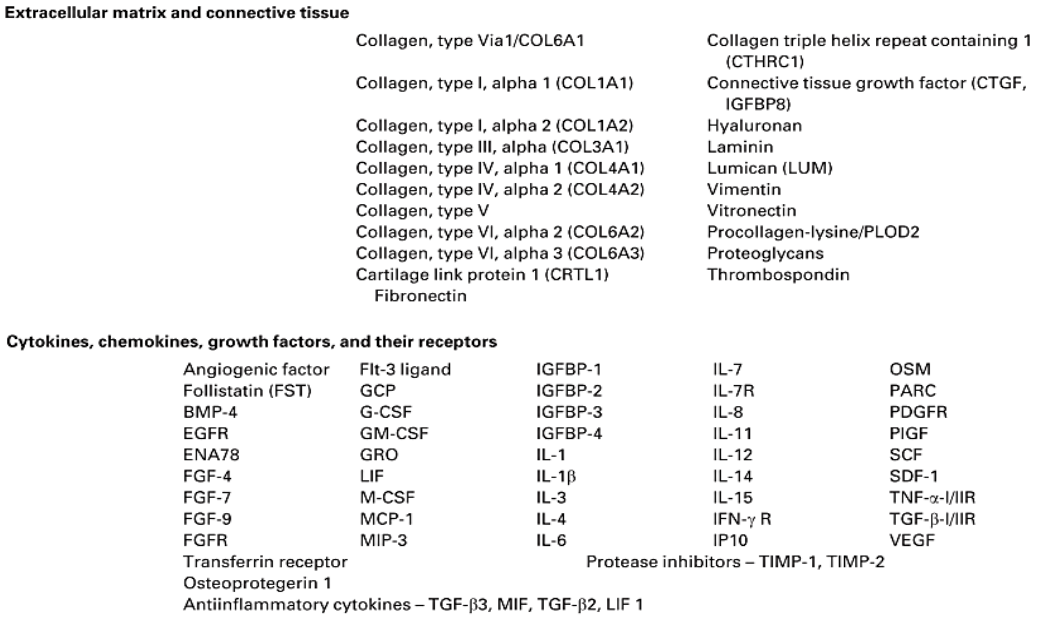

Figure 5. Extracellular matrix proteins, cytokines, chemokines, growth factors and receptors identified in WJ-MSCs by proteomics analysis. Seven types of collagen where identified in MSCs, as well as Fibronectin, Laminin, Vitronectin and Proteoglycans; relative to cytokines and chemokines, a large set of them are expressed by MSCs and seem to have important roles in regenerative medicine (Adapted from Park et al., 2007[36])

Several studies have already analyzed Mesenchymal stem cells expressed proteins and the highly expressed proteins in MSCs can be grouped into several functional categories related with metabolism, developmental processes such as differentiation and proliferation, morphogenesis, extracellular matrix or cytoskeleton.A study reported a proteomics analysis of Wharton's Jelly-derived MSCs. Overall, 60% of the total proteome was identified as proteins belonging to the cytoskeleton compartment and involved in protein biosynthesis, folding and degradation. This fact evidenced the proliferative capacity

of WJ-MSCs as well as the ability of rapidly change their phenotype in response to external stimuli[38]. A majority of the proteins belonging to ECM components or cytokines indicated that both the unique ECM environment and specific cytokine induction constitute important elements of the functional states of MSCs.

1.4.1. EFFECT OF SECRETED MOLECULES BY MSCS

The effects of MSC-secreted bioactive molecules can be either direct or indirect: direct effects activate intracellular signaling while indirect effects activate the secretion of molecules by other cells. MSCs also secrete a number of bioactive factors that directly suppress immune recognition and the expansion of B and T cells[39]. Mesenchymal stem cells are the most well-studied and well-understood cell type in the field of stem cell therapy and whose secretome has been most extensively investigated for therapeutic applications. So far, a variety of pro-inflammatory, anti-inflammatory and pleiotropic cytokines, chemokines, angiogenic factors, growth factors, growth factor-binding proteins, extracellular matrix proteins and extracellular matrix remodeling enzymes have been identified in mesenchymal stem cell secretomes. A number of angiogenic stimulators and inhibitors have been identified in MSCs secretome like vascular endothelial growth factor (VEGF), IL-6, fibroblast growth factor-2 (FGF-2) which secretion can be modified by chemokines. VEGF, TGF-β (transforming growth factor beta) and IL-6 are usually secreted by MSCs that have been recruited by tumor cells. Furthermore, it was reported that the secretome collected from MSCs populations may induce differentiation into different neuronal phenotypes[40].Thus, stem cell secretome represents a novel, promising alternative or complement to cell-based regenerative medicine therapies.

Overall, it has been shown that MSCs secretome varies depending on the microenvironment. Eventually, the proteome of MSCs could also vary depending on the external stimuli sensed by cells. In the case of a pathology, MSCs secrete a range of different molecules; and cells under specific conditions are able to escape from their original fate[41]. When physical properties of the microenvironment change, such as stiffness, MSCs secretome seems to suffer some alterations. Previous reports[42] showed an enhancement of secretion of paracrine factors when cytoskeletal tension increased. Dynamic MSCs studies have been conducted in order to elucidate about the molecular interplay between contractile apparatus and physical/biochemical stimuli sensed and integrated by MSCs and how it can affect their secretory and proteomic profile. Nevertheless, the issue of how mechanical cues affect the secretome and/or the overall proteome of MSCs remains very poorly explored.

1.5. OBJECTIVES

Mesenchymal stem cells are of great interest in clinical studies, since they can be readily isolated and expanded, exhibit multi-lineage differentiation potential and are known to secrete several bioactive molecules.

Some studies suggest that MSCs response to alterations of physical properties of their environment by changing marker expression and secretion of molecules.

We proposed that by using distinct cell culture substrates — namely using softer or stiffer materials, associated with ECM proteins —, the secretome of MSCs may suffer modifications, as well as the protein composition found in the membrane and cytoplasmic fractions.

Globally, this project aimed to understand how mechanotransduction elements influence the proteome (secretome, membrane and cytosolic proteomes) of WJ mesenchymal stem cells.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. UMBILICAL CORD (UC) SAMPLES PROCESSING AND CRYOPRESERVATION

2.1.1. UMBILICAL CORD (UC) TISSUE PROCESSING

Fresh human umbilical cords (UCs) were obtained from Crioestaminal, S.A. (BiocantPark, Cantanhede, Portugal) after informed consent of the donors. Biological samples were always manipulated under aseptic conditions using a class-II biosafety cabinet (Thermo Heraeus KS12). Samples were stored at 4°C, in sterile 50 mL conical tubes (Orange) with antibiotics for 48h at maximum, before tissue processing. Each umbilical cord sample was washed with sterile phosphate buffered saline solution (PBS, Life Technologies) to remove blood. Then, the samples were cut with a scissor into sections of about 5 cm long. The sections were washed and a longitudinal section was made in order to remove the vein and arteries to avoid endothelial cell contamination and obtain only cells from the region of interest — the *Wharton's Jelly.* Once obtained, *Wharton's jelly* was cut into 2-5 mm fragments with the help of a scalpel and tissue dissection tweezers and forceps.

2.1.2. CRYOPRESERVATION OF *WHARTON'S JELLY* FRAGMENTS

Fragments of *Wharton's jelly* were collected to cryopreservation vials with a forceps and it was added 1 mL of freezing medium [composed of Fetal Bovine Serum — FBS (Life Technologies) supplemented with 10% (v/v) of Dimethyl Sulfoxide — DMSO (Sigma-Aldrich)]. The vials were frozen overnight at -80°C in a cryo-cooler (VWR) and then transferred to a liquid nitrogen cryotank (Statebourne).

2.2. PREPARATION OF CELL CULTURE SUBSTRATES

2.2.1. PRODUCTION OF POLYDIMETHYLSILOXANE (PDMS) HYDROGELS

PDMS, a silicon elastomer, was used to produce substrates with tunable mechanical properties and tissue-like stiffness[43]. Crosslinked networks of PDMS were prepared by mixing the silicon elastomer base and the curing agent (Sylgard 184; Dow corning, USA). Two different mass ratios of base elastomer: curing agent were produced; 10:1 and 40:1. For each mixture, 1 mL was poured into 21 cm^2 plates to create approximately 200 µm thick hydrogels for cell culture. All PDMS substrates were left

to degas using a vacuum apparatus (Vacusafe, Integra Biosciences) at room temperature (RT) for 40 minutes to remove air bubbles. Thereafter, PDMS was cured at 70°C for 4 hours in an incubator (Binder).

2.2.2. TREATMENT OF PDMS HYDROGELS

Since native PDMS surface limits cell adhesion due to high hydrophobic surface properties, cured PDMS hydrogels were chemically treated (as described below). The goal was to modify the PDMS surface in order to establish a more hydrophilic surface and to allow the establishment of covalent links between ECM proteins and the PDMS surface[43,44], mimicking an *in vivo* cellular microenvironment, in order to facilitate cell adhesion and proliferation.

One drawback of PDMS substrates is putative surface roughness, which can negatively influence the efficiency of the treatment and cell adhesion (REF). In order to increase the efficiency of surface modification and diminish roughness, 10:1 substrates were detached from the plate and the polished surface (the one that polymerized in contact with the bottom of the plate) was exposed to the chemical treatment before further functionalization. 40:1 substrates were less hydrophobic and did not show problems in terms of cell adhesion, hence the upper surface of the substrate was directly treated and functionalized.

The chemical treatment consisted of adding three different solutions to PDMS substrates: Solution 1- hydrogen peroxide (H_2O_2, Sigma), Hydrochloric Acid (HCl, Fluka) and miliQ water (mQ water); Solution 2- 10% (v/v) of 3-aminopropyltrimethoxisilane (3-APTMS, Alpha Aesar) in 96% (v/v) ethanol (EtOH, Merck); Solution 3- 3% (v/v) glutaraldehyde in PBS.

First, PDMS substrates were treated with solution 1. 10:1 substrates were treated with solution 1 in a volumetric proportion of 1:1:3 and 40:1 substrates were treated with the same solution in a volumetric proportion of 1:1:5. The reaction occurred during 5 minutes at RT and allowed the formation of silanol groups on the surface of PDMS (hence the surface became more hydrophilic). The need for different volumetric proportions of solution 1 lies in the fact that 10:1 substrates showed higher hydrophobicity and required a more concentrated H_2O_2:HCl solution. Next, all substrates were washed three times with abundant mQ water and treated with solution 2 during 30 minutes at RT (the silane groups of 3-APTMS reacted with the silanol groups on the PDMS surface, hence establishing covalent links)[43]. Afterwards, the hydrogels were washed three times with mQ water, 10 minutes each time with agitation. The last solution - solution 3 – was added and the reaction occurred during 20 minutes at RT.

The aldehyde (–CHO) functional groups of glutaraldehyde readily react with the amine ($–NH_2$) groups of 3-APTMS and provided strong covalent bonds, enabling the subsequent modification steps (2.2.3). Next, the plates were washed three times with agitation during 5 minutes each. The washes during the treatment are required to remove remaining reagents that could possibly compromise the subsequent reactions and threat cell culture. Once functionalized, substrates were exposed to ultra violet (UV) light for 30 minutes in an air flow cabinet to ensure sterilization.

2.2.3. CROSSLINKING OF ECM PROTEINS ON PDMS HYDROGELS

In vivo, the extracellular matrix (ECM) plays essential roles in cell behavior and development. According to the objectives of the present study, to culture stem cells on PDMS substrates, it is necessary to mimic the extracellular matrix.

For that end, a mixture of two of major structural proteins composing ECM, *in vivo*, was applied on PDMS hydrogels in order to coat its surface and allow cell adhesion[45]. Human plasma purified Fibronectin (FN) and rat tail type I Collagen, (both from Millipore) were mixed in PBS at a final concentration of 10 µg/ml and 17 µg/ml, respectively- Coating Solution. The coating solution containing FN and collagen was used to coat the functionalized PDMS substrates at a ratio of 143 µl per cm^2 of substrate surface, resulting in 1.4 $µg/cm^2$ of FN and 2.4 $µg/cm^2$ of collagen. Hydrogels were incubated with Coating Solution at 37°C for 3 hours, and then washed once with sterile PBS.

2.3. ISOLATION, EXPANSION AND CRYOPRESERVATION OF MSCS

2.3.1. ISOLATION AND CULTURE EXPANSION OF MESENCHYMAL STEM CELLS (MSCS) BY EXPLANT METHOD

To proceed to explant culture method, fragments were thawed at 37° C and washed with Alpha-MEM medium (Life Technologies) supplemented with 1% Penicillin/Streptomycin (Life Technologies) and 1% amphotericin B (Life Technologies). Groups of 30 fragments were transferred to 21 cm^2 tissue culture (TC) plate (Corning) and to 21 cm^2 PDMS hydrogel and left to dry for 30 minutes to promote the attachment of the fragments to the surface of the plate and hydrogel, respectively. When fragments attached, 6 mL of proliferation medium - Alpha-MEM (Life Technologies) supplemented with 10% (v/v) MSC-qualified Fetal Bovine Serum (FBS) (Hyclone), 1% (v/v) Penicillin/Streptomycin (Life Technologies) and 1% (v/v) amphotericin B (Life Technologies) - were added to culture plates/hydrogels. Once

fragments were immersed, they were incubated at 37ºC with 5% of CO2/95% air and 95% humidity until MSCs started migrating out of the umbilical cord fragments. After 10 days it was possible to observe first cells migrating. Fragments were kept in culture until well-defined colonies were obtained. Then, the fragments were removed and the cells were detached and seeded into new 21cm^2 TC plates or hydrogels, respectively, to homogenize the population of cells.

2.4. PASSAGING AND FREEZING

For the passaging and freezing procedures cells were washed twice with sterile PBS and detached with 0.05% (v/v) Trypsin-EDTA (Life Technologies) during 5 minutes at 37° C. Then, it was added Alpha-MEM supplemented with 10% (v/v) FBS (Life Technologies), 1% (v/v) Penicillin/ and 1% (v/v) Amphotericin B to inactivate trypsin and cells were collected to a conical centrifuge tube. Then followed a centrifugation (5810 R centrifuge, Eppendorf) at 290*g*, 22° C for 5 minutes. Afterwards, for passaging procedure, pellet was re-suspended with proliferation medium with the help of a serological pipette (Corning-Costar) and plated on a new TC plate at a density of 3,000 cells/cm^2. Cells were kept in an incubator (Binder C-150) at 37° C with 5% of CO2/95% air and 95% humidity until reach 80% of confluence. For freezing procedure, after centrifugation, pellet was re-suspended in 1 mL of FBS with 10% (v/v) DMSO and collected to a cryo-vial. The vials were frozen overnight at -80° C in a cryo-cooler and then transferred to a nitrogen cryotank.

2.5. PROLIFERATION KINETICS OF UC- MSCS

MSCs isolated from three independent cord samples were cultured in 21 cm^2 plates and PDMS hydrogels. Cells were cultured from P2 to P6 and counted once they reached 80% confluence at each passage. Initially, fragments of UCs were cultured in 21 cm^2 TC plates (P0) and homogenized in the same type of plates- Passage 1 (P1). At passage 2, homogenous populations of cells were transferred to 21 cm^2 TC plates and 21 cm^2 PDMS hydrogels (10:1 and 40:1) in a density of 3,000 cells/cm^2. Cells isolated on PDMS substrates were not part of kinetic study. Kinetic study was only applied to cells isolated on TCPS and then cultured on TCPS and transferred to 40:1 PDMS gels.

The population doubling (PD) rate was calculated at each passage using the equation $N_H/N_I=2^X$, or $[\log_{10}(N_H) - \log_{10}(N_I)]/\log_{10}(2) = X$, where N_I represents the number of cells plated at each passage, N_H the number of cells harvested at the end of each respective passage and X the population doubling (PD).

The PD for each passage was calculated and added to the PD of the previous passages to generate cumulative population doublings (CPD). In addition, the generation time (GT) – average time between two cell doublings — was calculated from P3 to P6 using the following formula: $X = [\log_{10}(2) \times \Delta t] / [\log_{10}(N_H) - \log_{10}(N_I)]$. The total number of cells (TNC) was determined at each passage by cumulative counting of the cells once they reached a confluence of 80%, using the formula: $X = N_H \times B/N_I$, in which B represents the total number of cells in the previous passage. TNC accounts for the theoretical number of cells that could be obtained if no cells were discarded between each passage[46].

2.6. IMMUNOPHENOTYPIC CHARACTERIZATION OF UCM-MSCS

The immunophenotypic characterization of MSCs was performed before in collaboration with Centro Hospitalar da Universidade de Coimbra – Unidade de Gestão Operacional de Citometria. The immunophenotypic step was performed prior to cell culture. Cells were detached with accutase (LifeTechnologies) and the cell pellet was stained with monoclonal antibody (mAb) for surface protein antigens and, after an incubation period of 10 minutes in the darkness at room temperature (RT), washed with PBS. Then, cells were ressuspended in 250µL of PBS and immediately acquired in a FACSCanto II (BD) flow cytometer. The mAb used were conjugated with the following fluorochromes: fluorescein isothiocyanate (FITC), allophycocyanin (APC), phycoerythrin (PE), phycoerythrin-cyanine 7 (PECy7), krome orange (KO) and phycoerythrin-cyanine 5 (PerCPCy 5.5) The following monoclonal antibodies were used for the labelling: CD105 FITC (clone 2H6F11, Immunostep), CD90 APC (clone 5E10, BD Pharminogen), CD73 PE (clone AD2, BD Pharminogen), CD13 PECy7 (clone Immu103.44, Beckman Coulter), CD45 KO (clone J.33, Beckman Coulter) and CD34 PerCPCy 5.5 (clone 581, BD Biosciences).

2.7. MULTIPLEX CYTOKINE ANALYSIS

In order to analyze the concentration of cytokines on MSCs' conditioned media, an antibody-based multiplex analysis was performed using a Bio-Plex Pro 8-Plex Panel Assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions and as detailed below, using a Bio-Plex 200 system (Bio-Rad). The 8-plex panel evaluated the presence of the following analytes: GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α.

Conditioned media were obtained as described in II.8, except that after the 48h conditioning, media were collected (at least 100 µL of medium from each condition was obtained) and immediately centrifuged (at 290*g*, 4°C, for 5 minutes) to remove cell debris. Samples were frozen at -80°C until the analysis was performed Conditioned media were collected and centrifuged (290*g*, 4° C for 5 minutes) and 100 µL of medium of each platform was used to perform the assay.

Samples were thawed on ice and homogenized using a microplate agitator and then processed as follows (according to the kit's instructions). Samples were incubated at RT with agitation with the antibody-conjugated beads for 1 hour and then washed three times using the kit's Wash Buffer, followed by vacuum aspiration. The incubation with detection antibodies was performed for 30 min (RT) with agitation, followed by three washes as before. Finally, the incubation with the antibody conjugate streptavidin-phycoerythrin (SA-PE) was performed for 10 min (RT, with agitation), followed by three washes/aspiration cycles. The beads were re-suspended with Assay Buffer and data was acquired using a Bio-Plex 200 system. Acquisition and analysis was performed using the software Bio-Plex Manager 5 (BioRad).

2.8. SECRETOME AND SUBCELLULAR PROTEOME ANALYSIS

To proceed to secretome collection and subproteome fractionation, 3 million cells were seeded on 2 different platforms — TCPS (tissues culture plates) and 40:1 PDMS hydrogels — at a density of 6, 000 cells/cm^2. Cells were incubated at 37° C with 5% of CO_2/95% air and 95% humidity with proliferation medium until reaching approximately 50% of confluence. Next, the medium was discarded, cells were washed twice with PBS and once with Alpha-MEM supplemented with antibiotics to remove the remaining FBS from proliferation medium. Finally, to obtain conditioned medium, Alpha-MEM supplemented with antibiotics was added to the cells and incubated for 48 hours (using a CO_2 incubator as described above).

2.8.1 SECRETOME COLLECTION

After 48 hours of media conditioning, 100 mL of medium were collected from each platform and centrifuged at 290*g*, for 5 minutes at 4°C to remove cell debris. Then the secretome was concentrated using cut-off filters of 5 kDa (Vivaspin20, Sartorius) until 1 mL of concentrated conditioned media was obtained. The concentrated conditioned media were precipitated using Trichloroacetic acid (TCA) - Acetone. TCA was added to each sample to a final concentration of 20% (v/v), followed by an incubation at -80^{o}C and centrifugation at 20,000*g* for 20 minutes. Protein pellets were washed with ice-cold (-20°C) acetone, the pellets were solubilized in acetone, aided by ultrasonication, followed by a centrifugation at 20,000*g* for 20 minutes. The washed pellets were re-suspended in 2× Laemmli buffer (BioRad), aided by ultrasonication and denaturation at 95°C for 5min.

2.8.2. SUB-CELLULAR FRACTIONATION

To obtain cellular extracts, cells were washed with PBS and then incubated with extraction buffer (50mM Tris-HCl buffer pH 7.4 with protease inhibitors — Protease Inhibitor Cocktail tablets, Complete EDTA-free, from Roche). Next, cells were subjected to ultrasonication in water-bath (VibraCell 750 watts, Sonics®) with 40% amplitude with 30 second cycles (one second on followed by one second off and used for all procedures). After a 5 minutes centrifugation step at 4°C , 1,000*g* the supernatant was centrifuged at 126,000*g* at 4°C for 1 hour (Beckman Coulter)[47], and the pellet corresponding to the membrane-enriched fraction was solubilised in SDS sample buffer (1.7% SDS and 100mM DTT in 50mM Tris buffer at pH 6.8)[48]. Five volumes of -20°C acetone were added to each soluble fraction and were stored at -20°C to precipitate the protein content, which was then recovered by centrifugation at 4,000*g* for 30 minutes at 4°C and the protein pellets were washed with ice-cold (-20°C) acetone. Afterwards the pellet correspondent to the soluble fraction was re-suspended in SDS sample buffer [49,50]. Protein quantity was assessed using the Direct Detect Spectrometer (Milipore) according to the manufacturer's instructions, and 100 µg or the total amount protein (soluble or membrane fraction, respectively) were used from for LC-MS/MS analysis.

2.8.3. IN GEL DIGESTION AND SAMPLE PREPARATION

After denaturation, samples were alkylated with acrylamide and subjected to in gel digestion by using the short-GeLC approach[51]. Briefly, the entire sample was loaded in a "4–20% TGX Stain-Free Gel" (Bio-Rad) and subjected to a partially electrophoretic separation: 15 min at 110 V to allow the samples to enter into the gel. After SDS-PAGE proteins were visualized with Colloidal Coomassie Blue and the staining was performed as previously described in the work of Candiano and co-workers[52] with slight modifications[47].

The entire lanes were sliced into 3 parts and each part was sliced in small pieces and processed. Gel pieces were destained using the destaining solution (50 mM ammonium bicarbonate and 30% acetonitrile) followed by a washing step with water. Gel pieces were dehydrated on Concentrator Plus/Vacufuge® Plus (Eppendorf). Eighty (80) µL of trypsin (0.01 µg/µL solution in 10 mM ammonium bicarbonate) were added to the dried gel bands and left for 15 min, on ice, to rehydrate de gel pieces, after this period 25 µL of 10 mM ammonium bicarbonate were added and in-gel digestion was performed overnight at room temperature in the dark. After the digestion, the excess solution from gel pieces were collected to a low binding microcentrifuge tube (LoBind®, Eppendorf) and peptides were extracted from the gel pieces by sequential addition of three solutions of acetonitrile (ACN) in 1% formic acid (FA) (30%, 50%, and 98% of ACN, respectively). After the addition of each solution, the tubes were shaken in the thermomixer (Eppendorf) at 1050 rpm for 15 min and the solution was collected to the tube containing the previous fraction. The peptides mixtures were dried (preferentially not completely) by rotary evaporation under vacuum.

Before performing the LC-MS/MS analysis the peptide mixtures were subjected to SPE (solid-phase extraction) using OMIX tips with C18 stationary phase (Agilent Technologies) as recommended by the manufacture. Eluates were dried by rotator evaporation, avoiding to totally evaporate the samples and peptides mixtures were re-suspended to 30 µL in a solution of 2% ACN and 0.1% FA followed by vortex, spin and sonication in water bath (2 min with pulses of 1 second – 1 s sonication followed by 1 s break pulse –, at 20% intensity, in a sonicator VibraCell 750 watts, Sonics®). In order to remove insoluble material the peptide mixture was then centrifuged for 5 min at 14,000g and collected into the proper vial for LC-MS analysis.

2.8.4. SWATH ACQUISITION

Samples were analyzed on a Triple TOF™ 5600 System (ABSciex®) in two phases: information-dependent acquisition (IDA) was followed by SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra) acquisition on the same sample. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXP™ C18AR (300 µm ID × 15cm length, 3 µm particles, 120 Å pore size, Eksigent®) at 5µL/min. Peptides were eluted into the mass spectrometer with an acetonitrile gradient in 0.1% FA (5% to 35% ACN, in a linear gradient for 45 min), using an electrospray ionization source (DuoSpray™ Source, ABSciex®).

Information dependent acquisition (IDA) experiments were performed for each 3 peptides mixtures of the pooled samples composed by the combination of the biological replicates of each condition. The mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250ms, followed by up to 60 MS/MS scans (100–1500 m/z from a dynamic accumulation time – minimum 50 ms for precursor above the intensity threshold of 1000 – in order to maintain a cycle time of 3.3 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 25 seconds (mass spectrometer operated by Analyst® TF 1.6, ABSciex®). Rolling collision was used with a collision energy spread of 5. Peptide identification and library generation were performed with Protein Pilot software (v5.0, ABSciex®), using the following parameters: i) search against a database composed by *Homo sapiens* from SwissProt, and *malE*-GFP; ii) acrylamide alkylated cysteines as fixed modification; iii) trypsin as digestion type. An independent False Discovery Rate (FDR) analysis using the target-decoy approach provided with Protein Pilot software was used to assess the quality of the identifications and positive identifications were considered when identified proteins and peptides reached a 5% local FDR[51,53].

A single analysis of each sample was set for quantitative analysis by acquisition in SWATH mode. For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode [6] and the same chromatographic conditions used as in the IDA run described above. The SWATH-MS setup was designed specifically for the samples to be analyzed, to that a pool of all samples was analyzed in IDA mode in order to be used to create to adapt the SWATH windows to the complexity of the set of samples to be analyzed. A set of 60 windows of variable width (containing 1 m/z for the window overlap) was constructed covering the precursor mass range of 350-1250 m/z. A 200 ms survey scan (350-1500 m/z) was acquired at the beginning of each cycle for instrument calibration and SWATH

MS/MS spectra were collected from 100–1500 m/z for 50 ms resulting in a cycle time of 3.25 s from the precursors ranging from 350 to 1250 m/z. The collision energy for each window was determined according to the calculation for a charge +2 ion centered upon the window with a collision energy spread of 15.

A specific library of precursor masses and fragment ions was created for each sample type (secretome, membrane and soluble fractions) by combining the TCPS and PDMS files from the IDA experiments, and used for subsequent SWATH processing. Libraries were obtained using Protein Pilot™ software (v5.0, ABSciex®) with the same parameters as described above.

Data processing was performed using SWATH™ processing plug-in for PeakView™ (v2.0.01, ABSciex®), briefly peptides were selected automatically from the library using the following criteria: (i) the unique peptides for a specific targeted protein were ranked by the intensity of the precursor ion from the IDA analysis as estimated by the ProteinPilot™ software, and (ii) Peptides that contained biological modifications and/or were shared between different protein entries/isoforms were excluded from selection. Up to 15 peptides were chosen per protein, and SWATH™ quantitation was attempted for all proteins in library file that were identified below 5% local FDR from ProteinPilot™ searches. In SWATH™ Acquisition data, peptides are confirmed by finding and scoring peak groups, which are a set of fragment ions for the peptide.

Target fragment ions, up to 5, were automatically selected and peak groups were scored following the criteria described in Lambert et al[54]. Peak group confidence threshold was determined based on a FDR analysis using the target-decoy approach and 1% extraction FDR threshold was used for all the analyses. Peptide that met the 1% FDR threshold in at least one samples were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window adapted for each set of sample: 3 min in the case of the samples from the soluble fraction, and 4 and 5 minutes for membrane and secretome samples, respectively.

The levels of the human proteins were estimated by summing all the transitions from all the peptides for a given protein (an adaptation of[55]) followed by two steps of data normalization: (1) normalized to the internal standard (*malE*-GFP) followed by (2) a normalization using the sample total intensity.

2.8.5. BIOINFORMATIC TOOL FOR DATA ANALYSIS

PANTHER Classification System (http://www.pantherdb.org/) was performed for Gene Ontology analysis. GeneOntology enrichment analysis was performed for proteins identified

using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – GOrilla (http://cbl-gorilla.cs.technion.ac.il/). In order to identify and collect information about the proteins that were found, UniProt (http://www.uniprot.org/) was used. Venn graph were generate by BioVenn web application (http://www.cmbi.ru.nl/cdd/biovenn/).

2.9. STATISTICAL ANALYSIS

Statistical analysis of kinetitcs proliferation and multiplex-cytokine assay was performed by Mann-Whitney test, where statistically significant differences are indicated (*P<0.05) using the software GraphPad Prism 6.

Proteomics data was presented as the mean fold change of PDMS over the respective TCPS sample for the 3 biological replicates (umbilical cords from 3 different donors). Statistical analysis was performed in IBM® SPSS® Statistics Version 22 for all the proteins that presented PDMS/TCPS ratios with coefficient of variation below 30%. Data normality was accessed by the Shapiro-Wilk test performed in InfernoRDN and a one sample *t*-student test against a theoretical value of 1 was used to test for statistical meaningful variations. Statistical significance was considered for p-value<0.05.

CHAPTER 3

RESULTS

3. RESULTS

3.1. POLYDIMETHYLSILOXANE (PDMS) HYDROGELS FUNCTIONALIZATION

Cell adhesion and its relationship with the substrate is usually influenced by the substrate properties, such as biomolecular/biochemical composition and rigidity. Cell interactions with ECM proteins are vital to many biological processes and matrix proteins have been used for coating substrates for *in vitro* cell culture. Proteins such as FN and collagen have an important role on cell adhesion and ECM-cell contacts[56]. Moreover matrix rigidity also influences cell behavior. PDMS has been used in cell culture due to its properties: it is a biocompatible substrate, therefore it can be used for long-term cell culture, and its tunable properties allow to mimic different *in vivo* conditions.

Hence, PDMS hydrogels were fabricated as described (2.2.1), chemically functionalized (2.2.2) and covalently conjugated with ECM proteins (fibronectin and collagen I). The effectiveness of the developed biocompatible substrate could be demonstrated by cell adhesion and proliferation identified by phase-contrast microscopy (Figure 6).

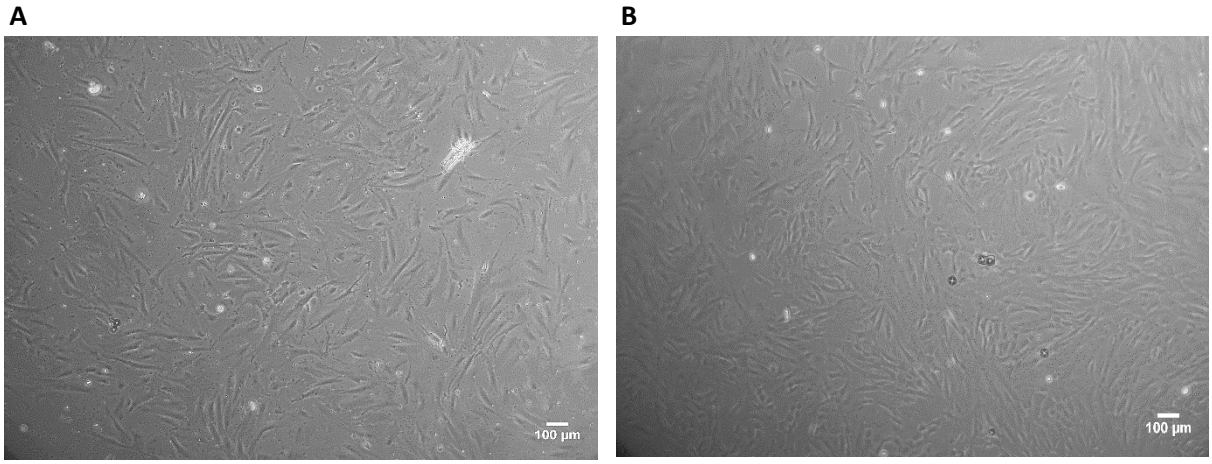

Figure 6. MSCs in culture on two distinct substrates. *(A)* 40:1 PDMS and *(B)* TCPS at day 4. *(A)* Phase-contrast microscopy images (5X objective) representative of the effectiveness of chemical modification of PDMS surface as well as of the coating proteins. Scale bar corresponds to 100 µm.

3.2. ISOLATION AND CULTURE EXPANSION OF MESENCHYMAL STEM CELLS (MSCS) BY THE EXPLANT METHOD

Mesenchymal Stem Cells (MSCs) can be isolated from several tissues. However, due to their high frequency in the Umbilical Cord (UC), more specifically, in the Wharton's Jelly, the latter constitutes a promising alternative source of MSCs once the process of MSCs collection from this tissue is simple, painless and non-invasive. Additionally, UC is discarded after birth and it does not raise many ethical constrains when compared with other sources. These tissues have also a low probability of viral contamination and the existence of public and private banks makes them more accessible for further use in heterologous and autologous transplants.

3.2.1. ISOLATION AND CULTURE OF MESENCHYMAL STEM CELLS ON DISTINCT SUBSTRATES (TCPS AND 40:1 PDMS)

Human MSCs were isolated from three umbilical cords, as described in the Materials and Methods section. At the end of 10 days in culture, fragments of Wharton's Jelly were still attached to the culture plates and showed cells migrating from the tissue. Fragments cultured on PDMS substrates (10:1 and 40:1) showed first cells migrating after 15 days in culture. Colonies of cells displaying a MSCs-like phenotype and with spindle-shaped morphology could be identified by phase-contrast microscopy (Figure 7).

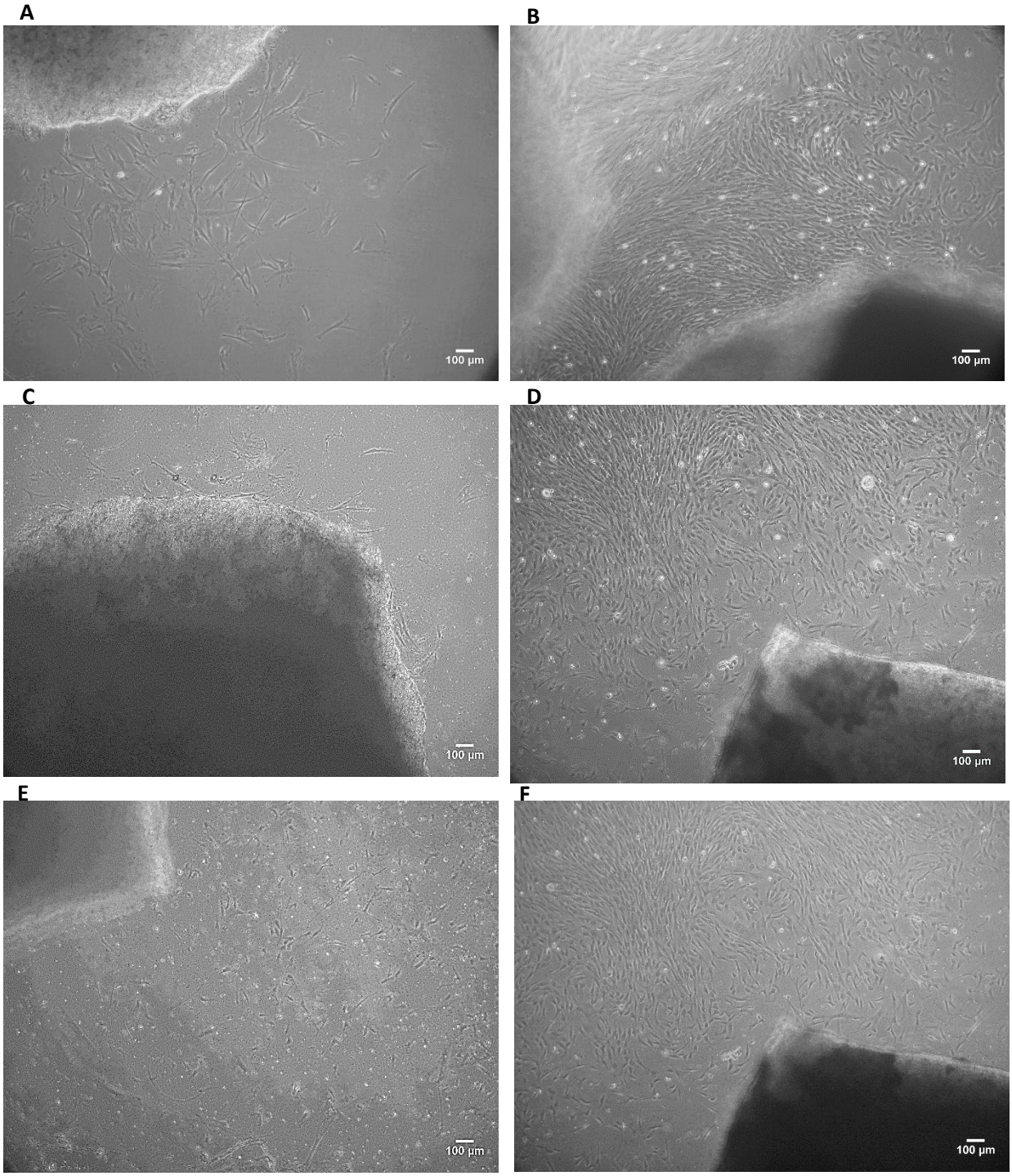

Figure 7. Wharton's Jelly explants culture and MSC-like cells migration. Phase-contrast microscopy images (5X objective) representative of MSCs isolated on ***(A), (B)*** *TCPS*; ***(C),(D)*** 10:1 PDMS*;* ***(E),(F)*** 40:1 PDMS*.* Left images – ***(A), (C)*** *and* ***(E)*** represent first cells migrating out of the explants while ***(B), (D)*** and ***(F)*** represent well defined colonies.

The cells cultured on TCPS formed compact colonies by the end of 14-16 days *in vitro*, while cells cultured on 10:1 and 40:1 PDMS substrates only formed compact colonies after 20 days in culture. Then the cells were dissociated using trypsin and reseeded on their respective substrate rigidity to homogenize the population (P1). After the first passage, the cells were constituted by a homogeneous population with MSC-like morphology. Cells isolated on both PDMS substrates (10:1 and 40:1) were cryopreserved. The cells cultured on TCPS were expanded on TCPS and 40:1 substrates from passage 2 until passage 6.

3.3. IMMUNOPHENOTYPIC CHARACTERIZATION OF UC-MSCS

Flow cytometry analysis demonstrated that the cells isolated from the WJ were positive for CD13, CD73, CD90 and CD 105 and negative for CD34 and CD45. This analysis **(Figure 8)** confirmed that cells isolated from Wharton's Jelly region presented the minimal criteria to be defined as MSCs according to the International Society for cellular Therapy[23] .

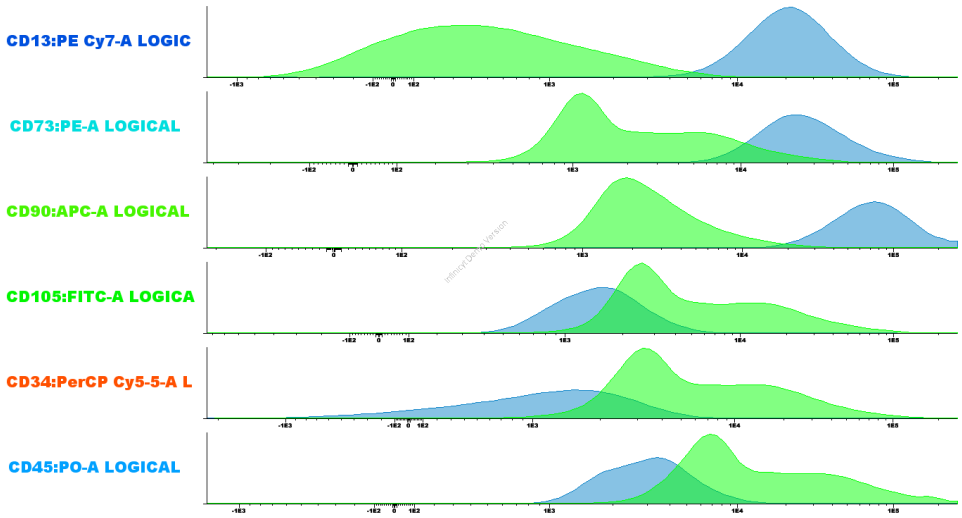

Figure 8. Representative immunophenotype of Wharton's Jelly MSCs. Cells were labeled with antibodies (as indicated) and analyzed by flow cytometry. Labeled MSCs (blue) showed positive expression of CD13, CD73, CD90 and CD105 and negative expression of CD34 and CD45. In green are represented the histograms of unlabeled cells.

3.4. PROLIFERATION KINETICS OF UC-MSCS

As indicated before, MSCs were isolated and cultured on TCPS and at Passage 2 (P2) they were transferred to 40:1 PDMS substrates in parallel to TCPS. MSCs were cultured at the same time on both substrates and their growth kinetics was analyzed as depicted on **Figure 9.**

As referred on the Materials and Methods section (2.5) the population doubling (PD) rate was calculated at each passage using the equation $N_H/N_I=2^X$, or $[\log_{10}(N_H) - \log_{10}(N_I)]/\log_{10}(2) = X$, where N_I represents the number of cells plated at each passage, N_H the number of cells harvested at the end of each respective passage and X the population doubling (PD). The PD for each passage was calculated and added to the PD of the previous passages to generate cumulative population doublings (CPD). In addition, the generation time (GT) – average time between two cell doublings — was calculated from P3 to P6 using the following formula: $X = [\log_{10}(2) \times \Delta t]/ [\log_{10}(N_H) - \log_{10}(N_I)]$. The total number of cells (TNC) was determined at each passage by cumulative counting of the cells once they reached a confluence of 80%, using the formula: $X = N_H \times B/N_I$, in which B represents the total number of cells in the previous passage. TNC accounts for the theoretical number of cells that could be obtained if no cells were discarded between each passage[46].

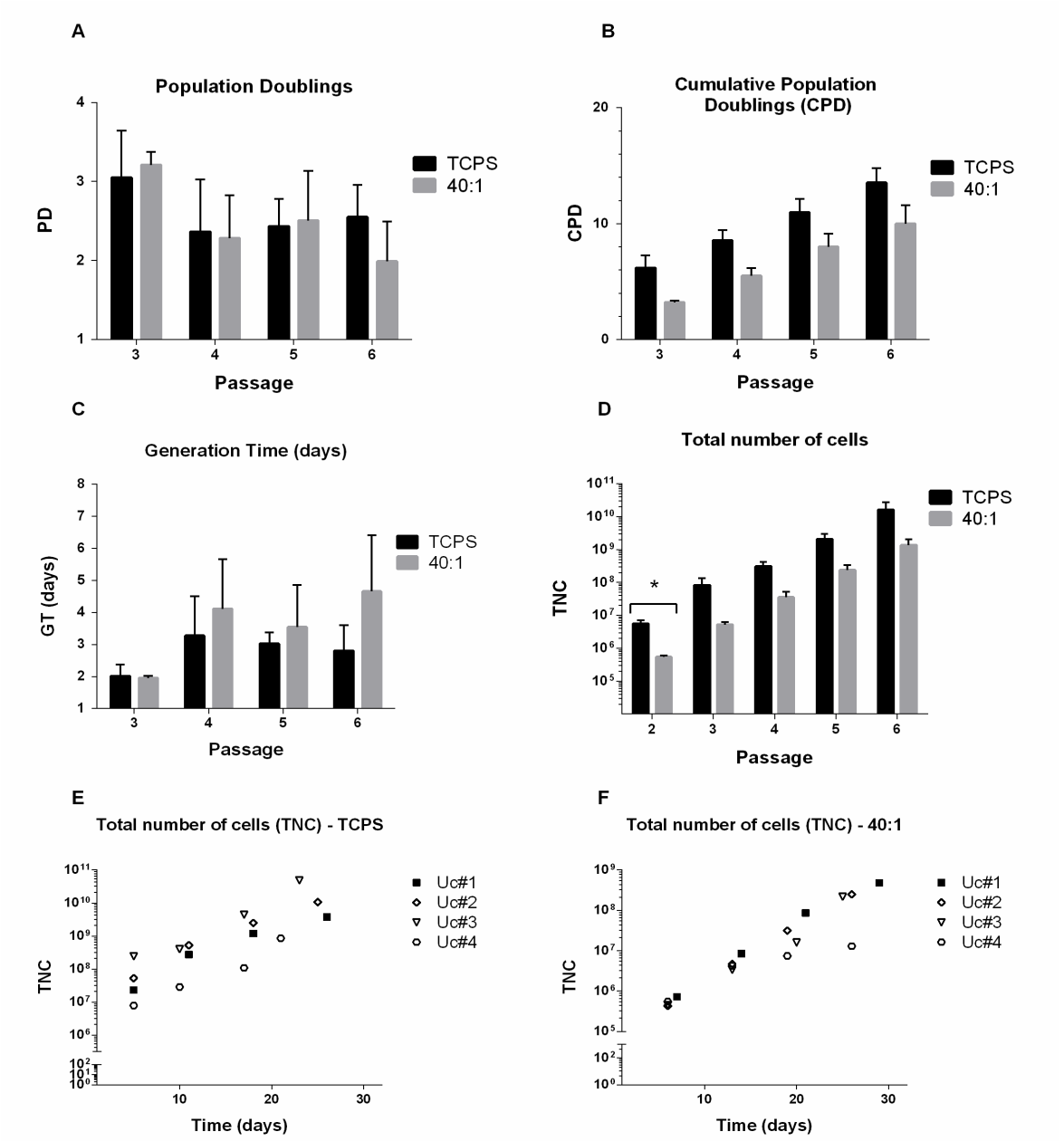

Figure 9. Proliferation kinetics of UC-MSCs cultured on two distinct substrates. Cells were passaged and counted once they reached around 80% confluence and the ***(A)*** population doubling (PD) was determined. ***(B)*** Cumulative population doubling (CPD) was determined by adding the calculated PD to PD of the previous passages; ***(C)*** Generation time (GT). ***(D)*** Total number of cells of each passage was calculated, during 6 passages or ***(E),*** (***F)*** over the time. Bars represent mean ± SEM of at least three independent experiments using cells obtained from different donors. Statistical analysis was performed by Mann-Whitney test using the software GraphPad Prism 6. Statistically significant differences are indicated (**P*<0.05).

As observed in Figure 9, between passage 3 and 6 (P3-P6), TCPS samples had, on average, doubled the population 9.81 ± 1.58 (SEM) times during which the generation time ranged from 1.88 ± 0.32 (SEM) to 3.27 ± 1.23 (SD) days. During the same passages, PDMS substrates had doubled the population on average 6.68 ± 1.48 (SEM) times with generation time varying from 1.95 ± 0.07 (SEM) to 4.65 ± 1.75 (SEM) days. The total number of cells (TNC) could be evaluated from passage 2 since this parameter does not need previous information. This value corresponds to the total number of cells that could be obtained if no cells were discarded during these passages, 26 days for TCPS, was $1.61 \times 10^{10} \pm 1.12 \times 10^{10}$ (SEM). For 40:1 substrates, the total number of cells was $1.37\times 10^{9} \pm 6.44 \times 10^{8}$ (SEM) during 29 days, corresponding to the same TCPS passages.

PDMS substrates showed lower CPD compared with TCPS which means that during the same passages the population doubled fewer times. The GT for both substrates seemed to suffer an increase after passage 3. However, on PDMS, it was observed a tendency to increase from this point until P6. This means that the time between two cell doublings increased for cells cultured on the referred substrate and suggests that soft substrates may influence cell proliferation.

Although both substrates achieved a clinically relevant number of cells (superior to 1×10^{9}), PDMS substrates presented a lower value for this parameter. For all the evaluated parameters, a statistical analysis was performed by Mann-Whitney test using the software GraphPad Prism 6. Data were compared regarding that biological variability was included, since cells were from 4 different donors. Besides that, a comparison between conditions (distinct substrate) was performed for all growth kinetics parameters and the only statistically significant difference (*P<0.05) observed was the TNC of PDMS and TCPS at passage 2 (Figure 9.D).

3.5. Multiplex cytokine analysis

To investigate whether differences were observed in the cytokine secretory profile of MSCs when substrate composition changed, an antibody-based multiplex cytokine assay was performed. Cells were cultured on TCPS or 40:1 substrates for approximately 14 days. Then, cells were maintained on the same substrates for 48 hours in serum-free medium and the respective conditioned media were analyzed. Different cytokines were evaluated and the results are shown in Figures 10-12. Regarding the raw data (pg/mL), results suggest a tendency for the decrease of IL-8, TNF-α, IL-6 and IL-4 secretion when cells

were cultured on softer substrates (40:1 PDMS), as compared to stiff TCPS. However, only IL-6 secretion decreased significantly (Figure 10).

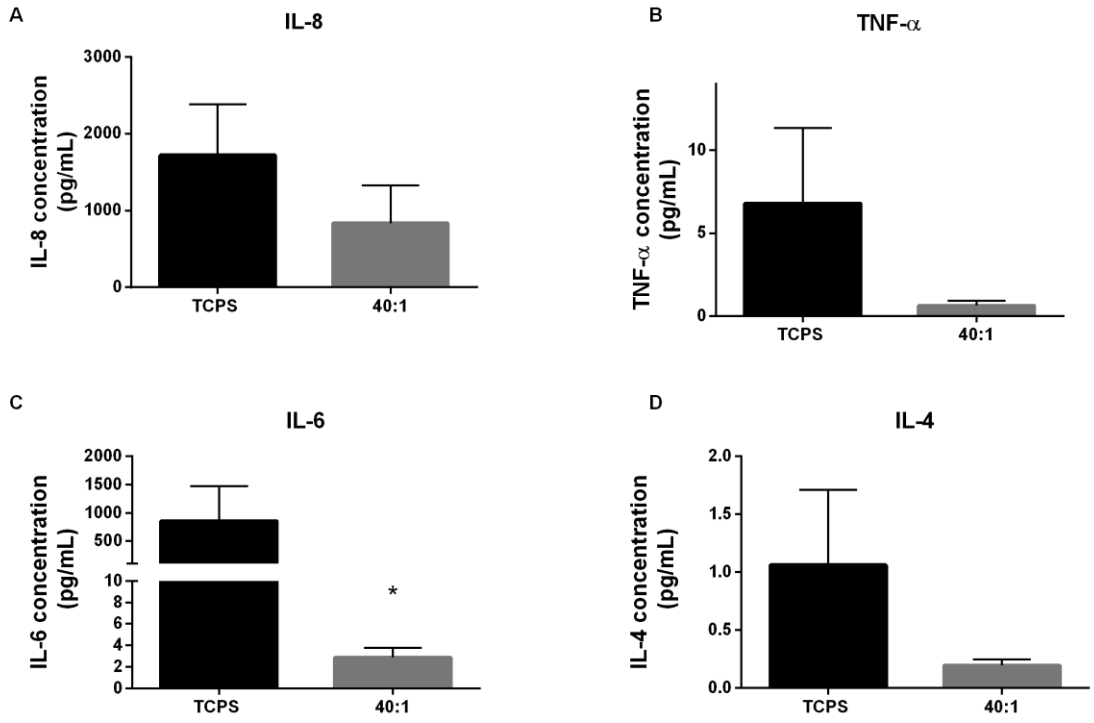

Figure 10. Evaluation of cytokine secretion by MSCs cultured on distinct substrates. Graphics represent cytokine concentration (pg/mL) quantified in media conditioned by MSCs maintained during 48h in serum-free medium on TCPS or 40:1 PDMS substrates, as indicated. Analysis was performed for ***(A)*** IL-8, (***B***) TNF-α, (***C***) IL-6 and ***(D)*** IL-4**.** Bars represent mean ± SEM of at least three independent experiments using cells obtained from different donors. Statistical analysis was performed by Mann-Whitney test using the software GraphPad Prism 6. Statistically significant differences are indicated (**P*<0.05).

In Figure 11.A is represented the final total cytosolic protein concentration after cells were cultured on 40:1 PDMS and TCPS substrates for 48 hour in serum free medium. As it is observed, there are no significant differences in protein concentration (mg/mL) obtained from cells maintained on the distinct substrates. The total protein quantification was used as a normalization factor of cellular content to more accurately compare MSCs cytokine secretion profile on both substrates. Similar to what was observed before (Figure 10), the normalized results showed a tendency for decreased secretion of all evaluated cytokines (IL-8, TNF-α, IL-6 and IL-4) when cells were cultured on softer 40:1 PDMS substrates (Figure 11 B-E). Nevertheless, the differences observed were not statistically significant.

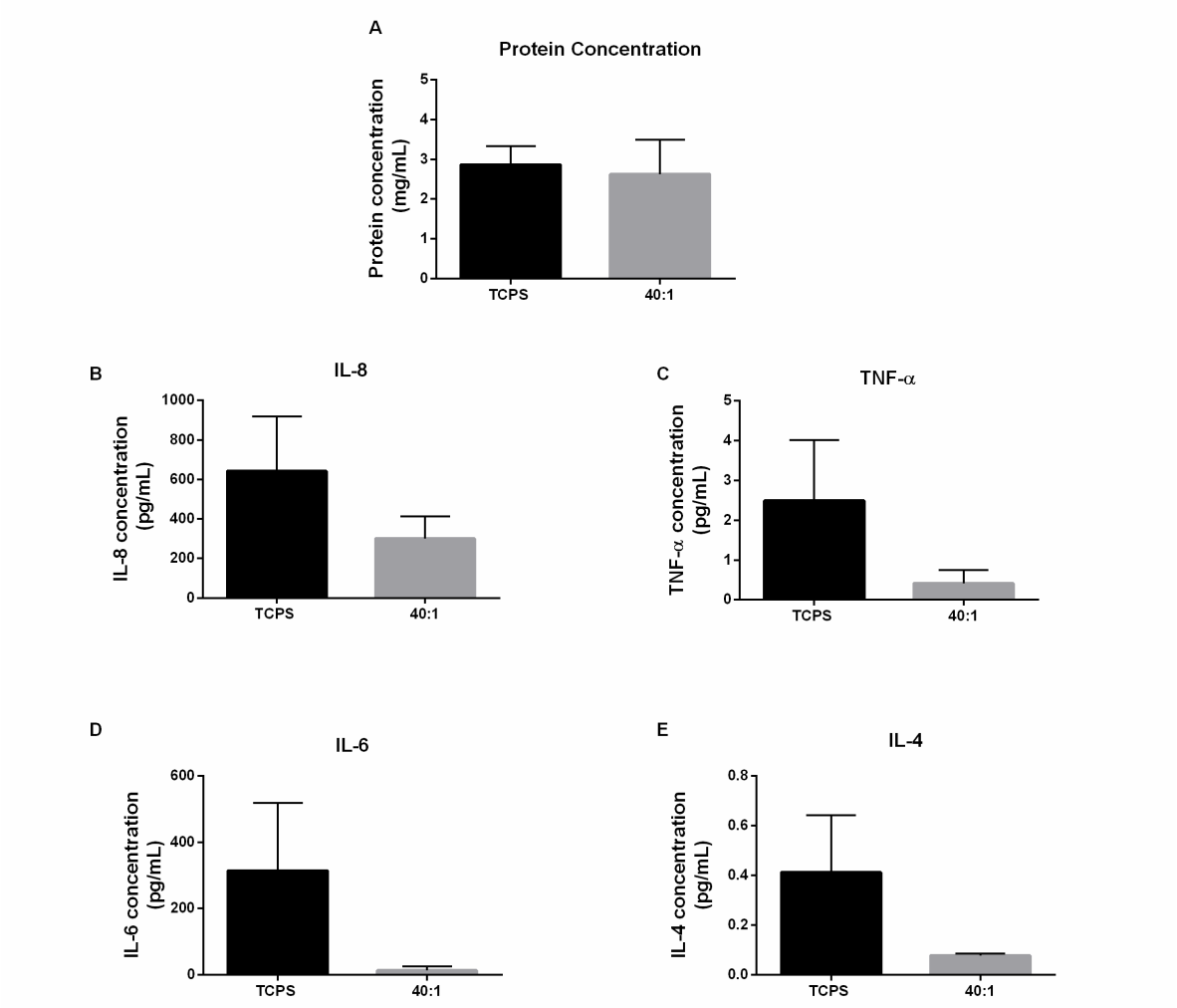

Figure 11. Evaluation of cytokine secretion by MSCs cultured on distinct substrates normalized to the final cellular cytosolic protein content. ***(A)*** Quantification of protein concentration (mg/mL) obtained from cells maintained on TCPS or 40:1 PDMS substrates. Protein quantification was performed by Direct Detect system. Bar represent mean ± SEM of cytosolic protein content from cells obtained from at least three independent samples. ***(B)-(E)*** Graphics represent cytokine concentration (pg/mL) quantified in media conditioned by MSCs maintained during 48h in serum-free medium on TCPS or 40:1 PDMS substrates, as indicated. Analysis was performed for ***(A)*** IL-8, ***(B)*** TNF-α, ***(C)*** IL-6 and ***(D)*** IL-4. Bars represent mean ± SEM of at least three independent experiments using cells obtained from different donors. Statistical analysis was performed by Mann-Whitney test using the software GraphPad Prism 6. Statistically significant differences are indicated (*P<0.05).

In order to reduce variability introduced by the fact that independent umbilical cord samples were used

in this study, implying higher biological variation, cytokine secretion data obtained from cells cultured

on 40:1 substrates was divided by each respective control (TCPS). This normalization step revealed a significant decrease of IL-6 and TNF-α secretion by cells cultured on 40:1 PDMS substrates (Figure 12). No other significant differences were found.

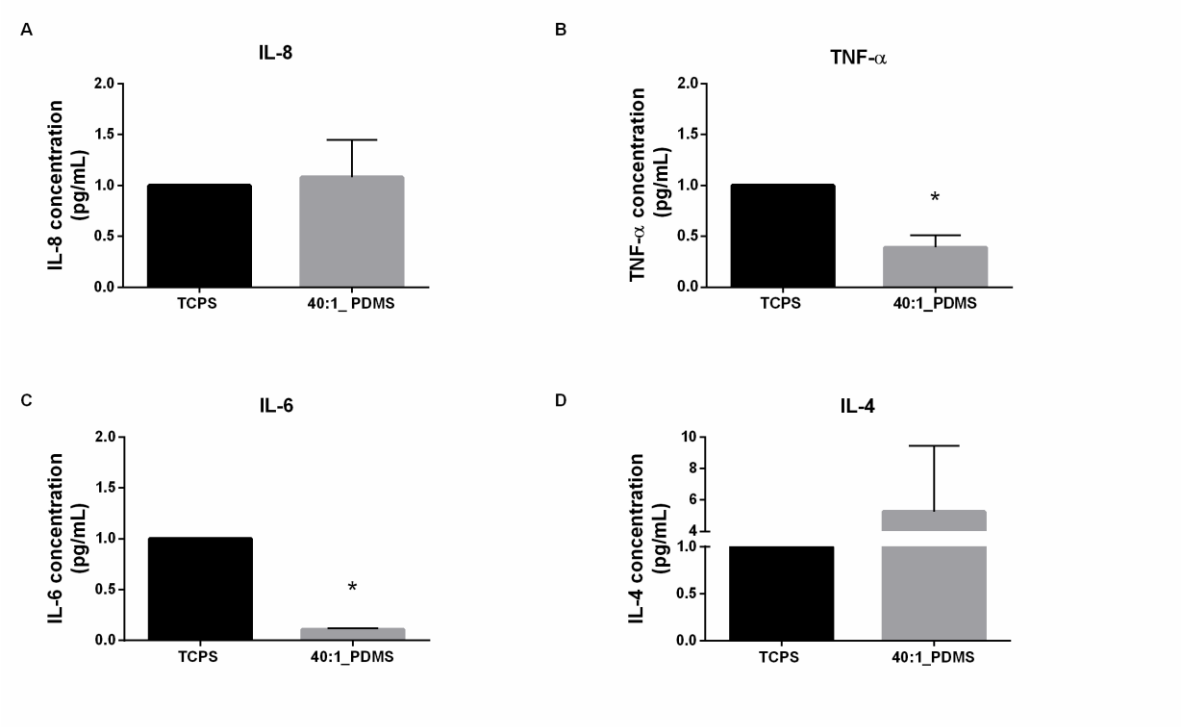

Figure 12. Fold change (relative to TCPS) of cytokine secretion (normalized to total cytosolic protein content) by MSCs cultured on 40:1 PDMS substrates. Graphics represent cytokine concentration (pg/ mL) quantified in media conditioned by MSCs maintained during 48h in serum-free medium on TCPS or 40:1 PDMS substrates, as indicated. Analysis was performed for *(A)* IL-8, (*B*) TNF-α, (*C*) IL-6 and *(D)* IL-4. Bars represent mean ± SEM of at least three independent experiments using cells obtained from different donors. Statistical analysis was performed by Mann-Whitney test using the software GraphPad Prism 6. Statistically significant differences are indicated (**P*<0.05).

3.6. SECRETOME AND SUBPROTEOME ANALYSIS

This study aimed to understand how substrate characteristics could influence Mesenchymal stem cell subproteomes (secretome, soluble and membrane proteomes). MSCs were maintained in culture as described (2.8.1) on two distinct substrates – TCPS representing a standard condition, and 40:1 PDMS substrates representing a biochemically and mechanically distinct culture condition. Conditioned media

obtained from cells on culture conditions were collected and concentrated as indicated (2.8.1). At the same time, subproteome fractionation was performed in order to study the influence of substrate composition in the different cell compartments—cytosol and membrane.

3.6.1 GENE ONTOLOGY ENRICHMENT ANALYSIS

In order to understand the protein profile of each fraction (Secretome, Membrane and Soluble fractions), a gene ontology (GO) analysis of cellular components was performed. For that, proteins identified in cells cultured on the TCPS and 40:1 PDMS substrates were combined and then compared with the total number of proteins found in the study.

Regarding the cellular component analysis, the results (Figures 13-15) confirmed that there was an enrichment of proteins bellonging to the secretome, cytosolic and membrane fractions of the cell, in their respective fractions. Figures 13-15 can be appreciated in more detail in the Supplementary data section (Figures S1-S3). Regarding the sub-cellular component analysis in more detail, results indicate that in the secretome fraction (the conditioned media of cells containing molecules secreted by MSCs) there was an enrichment of proteins that correspond to the extracellular region of the cell, as expected (Figure 13). Concerning samples that were obtained from the soluble fraction of the subproteome fractionation procedure, it was expected that an enrichment of proteins that belong cytosolic part of the cell occurred. The GO analysis confirmed an enrichment of this fraction in soluble proteins corresponding to the cytosolic region, with a p-value<10^{-9}, as represented (Figure 14).

Finally, the results also allowed to prove that samples obtained from the membrane-rich subcellular fraction were in fact enriched with proteins belonging to membrane region of the cell. More specifically, proteins were grouped in categories such as cell-substrate adhesion, focal adhesion and intrinsic component of membrane. This information is represented in red (Figure 15) with a p-value<10^{-9}.

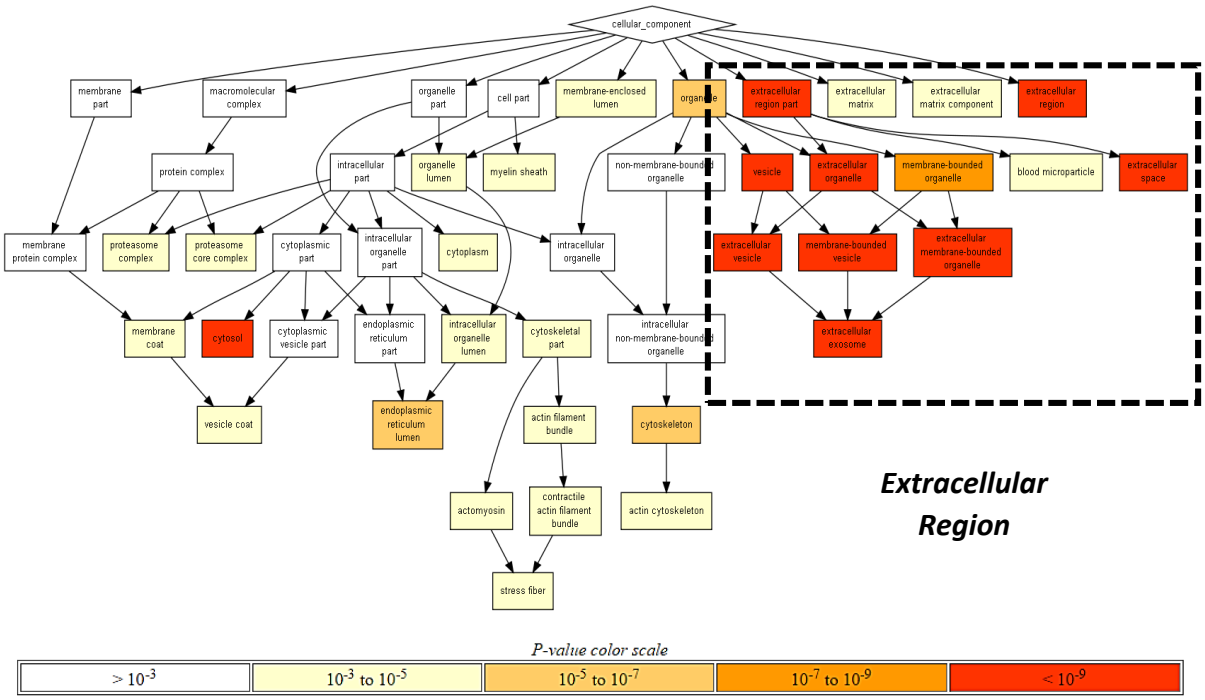

Figure 13. **Diagram representing the Gene Ontology (GO) Enrichment analysis at the cellular component level of the proteins identified in the Secretome fraction.** The proteins identified in the secretome fraction were highly enriched in proteins belonging to the extracellular region, as demonstrated by the p-values $<10^{-9}$ (in red), using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – GOrilla

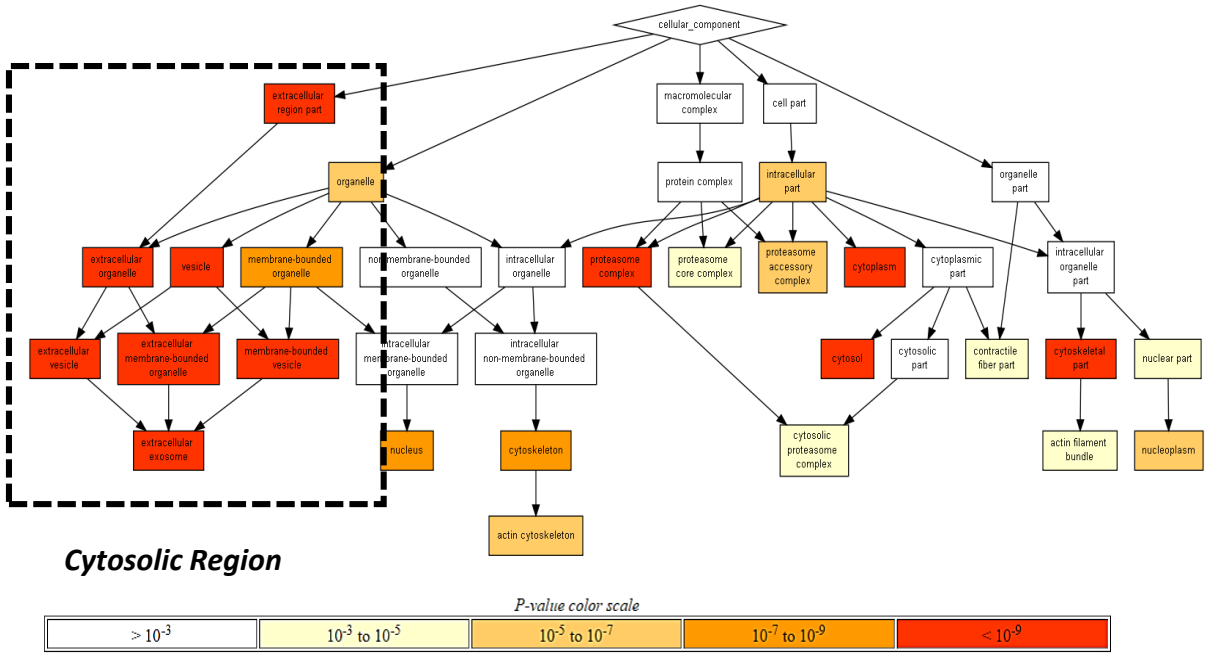

Figure 14. Diagram representing the Gene Ontology (GO) Enrichment analysis at the cellular component level of the proteins identified in the Cytosolic fraction. The proteins that showed higher enrichment were related to the extracellular region part and cytosolic part of the cell, demonstrated by a p-value<10^{-9}, using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – Gorilla.

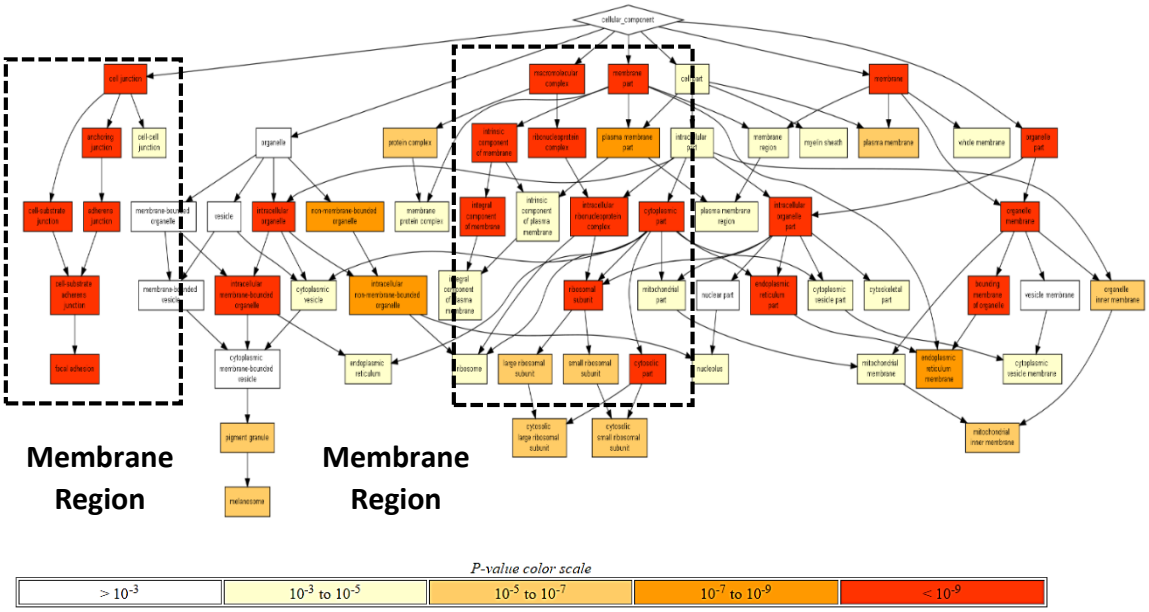

Figure 15. Diagram representing the Gene Ontology (GO) Enrichment analysis at the cellular component level of the proteins identified in the in membrane fraction. The proteins belonging to the membrane region were the ones with higher enrichment, demonstrated by a p-value<10^{-9}, using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – Gorilla.

3.6.2. PROTEIN IDENTIFICATION

After verifying the fraction enrichment by gene ontology, a step of identification of proteins was performed. The total number of identified proteins in each cellular fraction was found and compared within both substrates under analysis (TCPS and 40:1 PDMS) in order to clarify whether the proteins were unique or shared by cells cultured on the distinct substrates. This comparison is depicted below (Figure 16) for all distinct fractions: secretome, soluble and membrane.

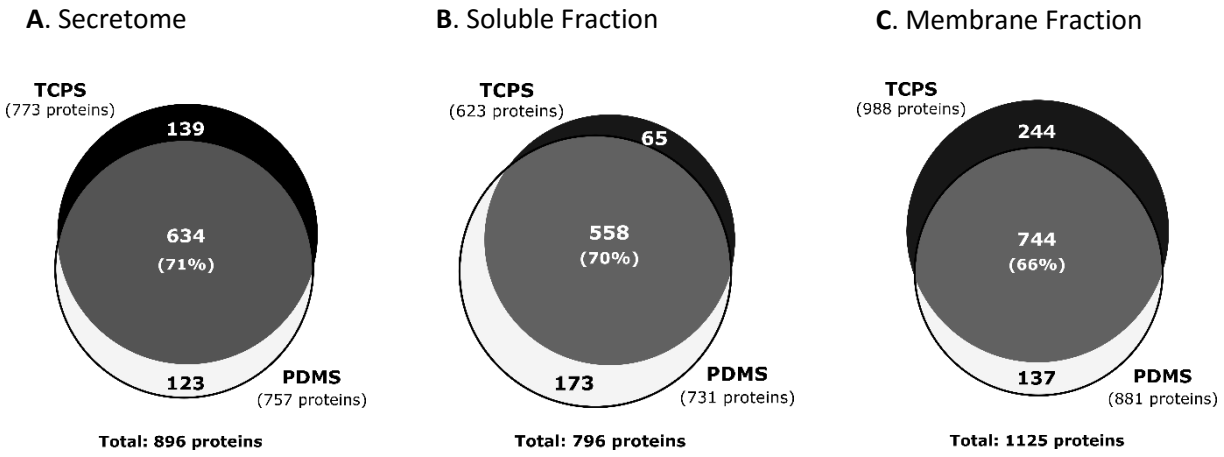

Figure 16. Venn diagrams illustrating the total number of proteins and the number of unique and shared proteins by cells cultured on distinct substrates (TCPS and 40:1 substrates). *(A)* Number of proteins of MSCs secretome; *(B)* Number of proteins of MSCs soluble fraction (cytosol); *(C)* Number of proteins of MSCs Membrane Fraction. Percentage numbers (71%, 70% and 66%) represent common proteins between both substrates.

The total number of identified proteins on each fraction ranged from 796 to 1125 (information available on digital format), whose highest value corresponds to membrane fraction identified proteins. Regarding the comparison between TCPS and 40:1 PDMS substrates, the protein identification revealed a similar number of identified proteins in all fractions and the majority of the proteins were common (66-71%) between both substrates, justifying the use of a quantitative method. In general, there was a slightly

increased number of unique proteins for cells cultured on TCPS, except in the soluble fraction (Figure 16.B) that showed a higher number of unique proteins on cells cultured on PDMS. More specifically, at the level of the secretome (Figure 16.A) a total of 896 proteins were found and within these, 634 were common for both distinct substrates (TCPS and 40:1 PDMS), which represent 71% of the total number of proteins and 120-140 proteins appeared to be exclusively secreted by cells cultured on a particular substrate.

Soluble Fraction presented a total number of 796 identified proteins from which 558 (70%) were common to both substrates and 173 were only found in cells cultured on 40:1 PDMS substrates. In contrast, a lower number of unique proteins were identified in cells cultured on TCPS (65). Membrane Fraction results (Figure 16.C) showed the identification of 1125 proteins, among which 744 (66%) were common between both substrates. A total of 880-990 proteins were found on each substrate and 137 proteins appeared exclusively on cells cultured on 40:1 PDMS substrates, while cells cultured on TCPS exhibited 244 unique proteins.

3.6.3. QUANTIFICATION OF PROTEINS OF ENRICHED FRACTIONS

Since the majority of proteins were common between substrates, proteins were quantified in order to detect alterations on protein levels.

Three independent umbilical cord samples were used, so in order to reduce the impact of biological variability, the data were analyzed through ratio of the protein levels of PDMS over TCPS (PDMS/TCPS) for each sample. Protein quantification (information available on digital format) revealed a total number of 635 proteins in the secretome, 633 for cytosolic fraction and 624 in case of membrane fraction. To identify the differences in the three types of sub-cellular proteomics profiles induced by the distinct substrates (TCPS and 40:1 PDMS), only the proteins with coefficient of variation (CV) below 30% (between independent samples) were used for statistical analysis. Thus, 253 proteins were found to have CV<30% for the secretome, while cytosolic and membrane fractions exhibited 356 and 227 proteins, respectively. From each cellular fraction, 79, 60 and 62 proteins (respectively) showed a p-value lower than 0.05 when comparing the quantitative data between cells cultured on PDMS 40:1 and TCPS, as summarized in Table 3.

Table 3. Comparison between the total number of proteins, number of proteins with CV<30% (within biological samples) and number of proteins with p-value<0.05 (between 40:1 PDMS and TCPS) in each cell fraction (secretome, cytosol and membrane).

Cell Fraction	Total Number of Proteins	Proteins with CV<30%	Proteins with p-value<0.05
Secretome	635	253	79
Soluble (cytosol)	633	356	60
Membrane	624	227	62

Within the proteins with p-value<0.05 for each enriched fraction, it was observed that some were down-regulated and others up-regulated when comparing cells cultured on 40:1 PDMS substrates relative to those on TCPS. Volcano plots represent whether proteins showed statistically significant differences among PDMS and TCPS substrates, for each enriched fraction. Moreover, the graphs also illustrate whether the proteins were up- or down-regulated on PDMS in comparison with TCPS (Figure 17. A-C).
In order to aid volcano plots interpretation, Table 4 contains the number of proteins varying on PMDS relative to TCPS, as well as how many were up- or down-regulated.

Table 4. Number of protein with significant variation (p-value<0.05). Number of proteins varying in each fraction, including number of up- and down-regulated proteins.

Cell Fraction	proteins with p-value <0.05	up-regulated proteins	down-regulated proteins
Secretome	79	27	52
Soluble (cytosol)	60	27	33
Membrane	62	19	43

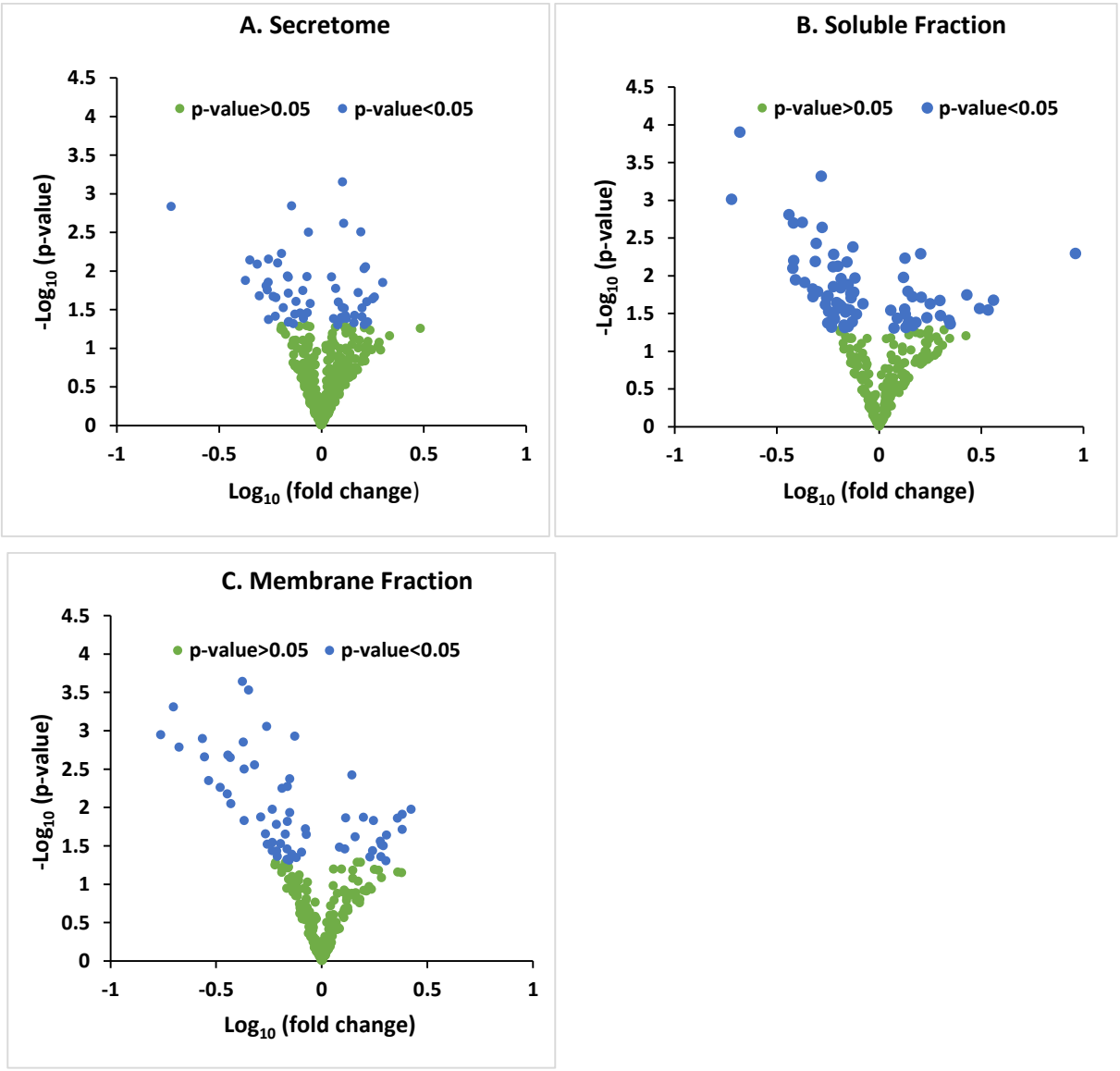

Figure 17. Volcano plots illustrating significant differences between proteins found in cells cultured on 40:1 PDMS substrates relative to TCPS. ***(A)*** Proteins respective to secretome; ***(B)*** proteins of soluble (cytosolic) fraction and ***(C)*** proteins of membrane fraction. ***(A), (B)*** and ***(C):*** Statistically significant differences are represented by blue plots, with a p-value<0.05. Green plots represent proteins that did not show significant differences relatively to TCPS (p-value>0.05).

Regarding the results, it was observed that the number of proteins found to vary within 3 analyzed fractions was 60-79, where the secretome showed a higher number of proteins varying on PDMS-cultured cells. In general, all three fractions presented an increased number of down-regulated than up-regulated proteins. Specifically, secretome showed 52 down-regulated and 27 up-regulated proteins;

soluble fraction appeared with 33 down-regulated and 27 up-regulated proteins and in membrane fraction were detected 43 down-regulated and 19 up-regulated proteins.

In order to understand how the referred proteins were assembled in terms of biological processes, a Gene Ontology analysis was used at the level of biological processes **(Figure 18)** and molecular functions **(Figure 19)**, and the results are depicted on PANTHER software charts.

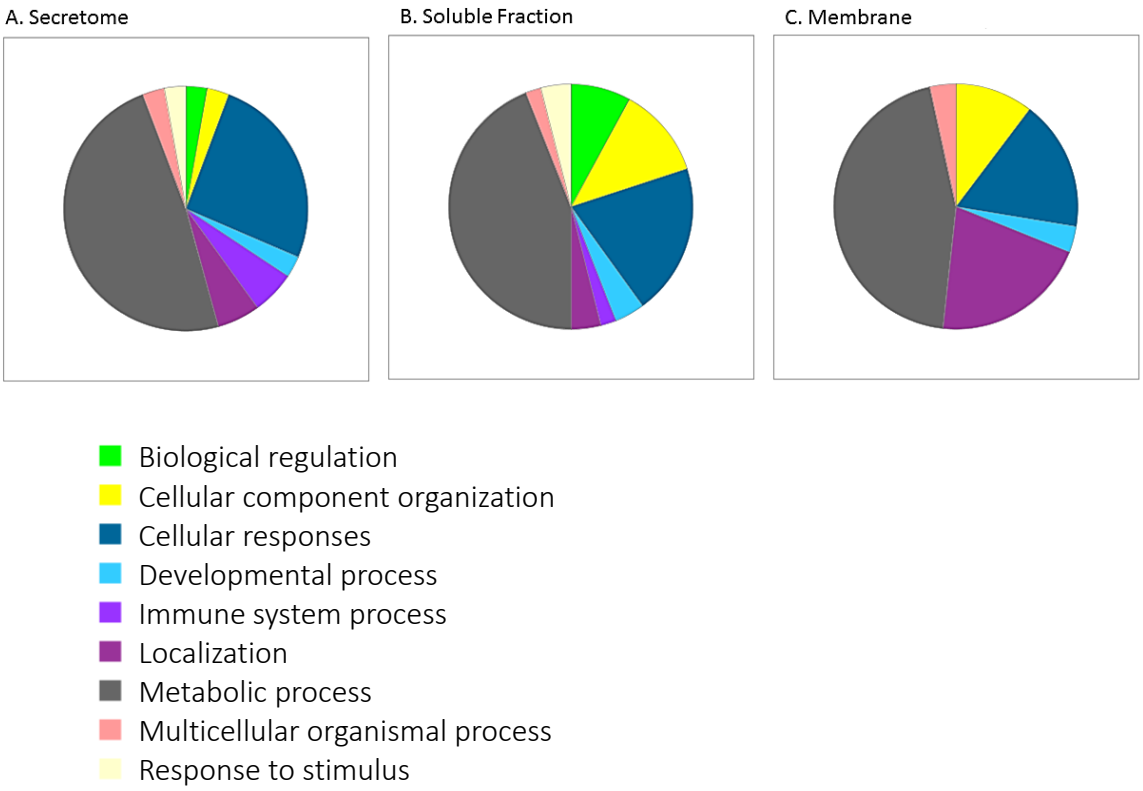

Figure 18. Pie charts illustrating the biological processes concerning the proteins up-regulated in cells cultured on 40:1 PDMS relative to TCPS, within each cellular fraction. (A) Secretome; **(B)** Soluble Fraction; **(C)** Membrane.

Regarding the pie charts **(Figure 18)**, it was clear that all of the 3 fractions showed a higher number of proteins involved in metabolic processes and cellular responses. The majority of the proteins within metabolic processes were related with primary metabolic processes. Cellular responses were related to cell communication, more specifically to cell-cell signaling. Among up-regulated proteins, some of them seemed to be related with cytoskeleton reorganization, more evidenced in soluble and membrane fraction.

Regarding the molecular function results **(Figure 19)**, proteins that were differentially expressed in the secretome, soluble and membrane fractions seemed to have common catalytic activity and to be involved in binding. Focusing on the secretome and soluble fractions, the majority of proteins had catalytic activity. Proteins belonging to this category seemed to have mainly oxireductase activity. Membrane fraction showed an increased number of proteins involved in structural molecule activity and transporter activity when compared with other fractions, which seems to be consistent with the expected functions of membrane-related proteins. Antioxidant activity is commonly represented in three fractions and it is related with peroxidase activity.

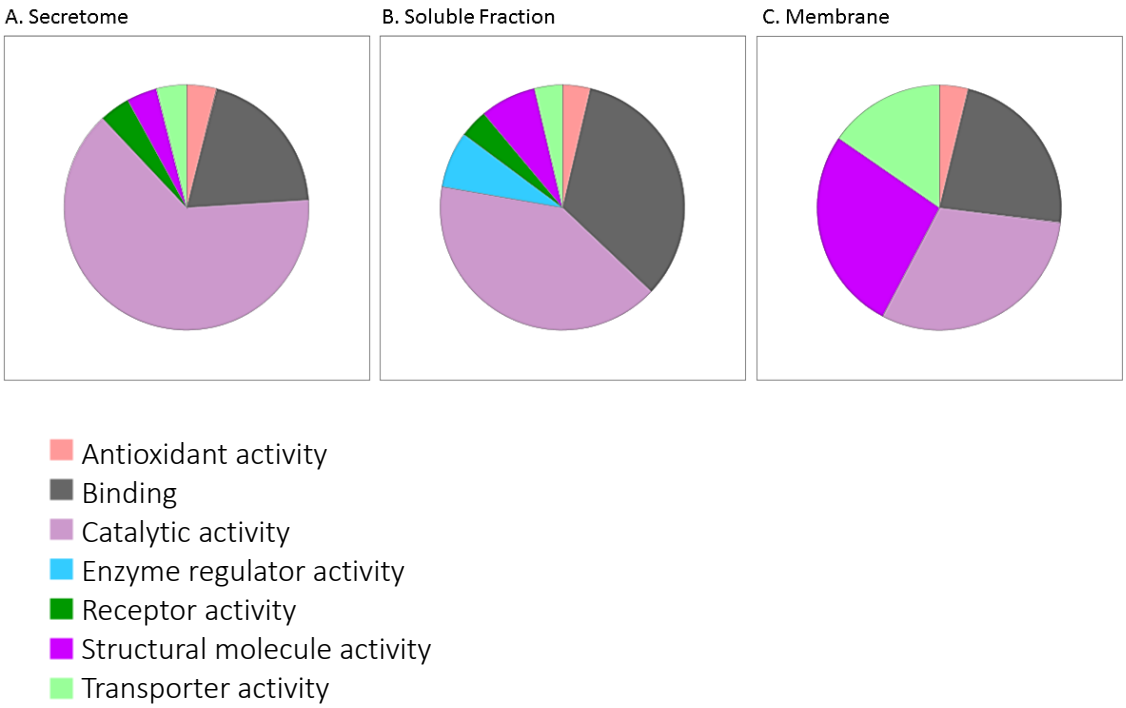

Figure 19. Pie charts illustrating the molecular functions of the proteins up-regulated in cells cultured on 40:1 PDMS relative to TCPS, within each cellular fraction. (A) Secretome; (B) Soluble Fraction; (C) Membrane.

Within all the up-regulated proteins, the one that showed the highest value of fold change was Superoxide Dismutase [Mn] (mitochondrial), which presented an up-regulation of approximately 9 fold

in secretome. Antioxidant activity was also evaluated in the remaining fractions and a total of 6 proteins related to this function were found (Table 5).

Table 5. Proteins with antioxidant activity found in secretome, soluble and membrane fraction.

Protein Name	Fraction	Mean	SD	%CV	p-value
1. Peroxiredoxin-1	Soluble fraction	1.27	0.012	1	0.001
2. Peroxiredoxin-5, mitochondrial	Membrane	1.89	0.261	14	0.028
3. Microsomal glutathione S-transferase 3	Membrane	1.904	0.339	18	0.044
4. Thioredoxin reductase 1, cytoplasmic	Secretome	2.231	0.46	21	0.044
5. Thioredoxin-dependent peroxide reductase, mitochondrial	Secretome	3.104	0.615	20	0.027
6. Superoxide dismutase [Mn], mitochondrial	Secretome	9.143	1.012	11	0.005

These results suggest that MSCs cultured on 40:1 PDMS substrates possess a higher anti-oxidant capacity than their counterparts cultured on TCPS. This observation is important, since one of the major features commonly attributed to MSCs is precisely their anti-oxidant and protective capacity in several biological contexts.

CHAPTER 4

DISCUSSION

4. DISCUSSION

MSCs are an exciting cell-based therapeutic candidate for use in regenerative medicine. Within MSCs based populations, WJ's cells have been described as an attractive population for cell therapy due to their characteristics such as trilineage mesodermal potential of differentiation, immune modulatory responses and due to the variety of secreted molecules with trophic activity. Additionally, these cells are obtained from an extraembryonic tissue at the time of birth, which suggests the existence of less genetic mutations when compared with cells from adult tissues[15].

The extracellular matrix (ECM), a major element of the extracellular niche, provides not only a scaffold for cellular support, migration and proliferation, but also acts as the surrounding microenvironment that influences growth, function and eventually fate of the cell by presenting biochemical and mechanical cues as well as binding soluble factors. Based on the literature, it is known that cells sense external mechanical stimuli through mechanotransduction mechanisms, translating biophysical cues into physiological responses [26,28,57]. The response can be at the level of morphology, proliferation, protein synthesis or even differentiation[58]. The current study presented an approach to understand the influence of biochemical and mechanical properties of ECM-like substrates over MSCs cultured *in vitro* and whether such elements influence their secretory profile and subcellular proteome.

To that end, MSCs were isolated from umbilical cord matrix – *Wharton's Jelly (WJ)* – and cultured on substrates with different characteristics, namely stiffness and ECM proteins.

Cells were cultured on tissue culture plates (TCPS) whose stiffness was at the level of GPa (gigapascal) and, on the other hand, on 40:1 PDMS substrates representing a softer culture condition with a described stiffness ranging between 30 to 40 kPa (KiloPascal)[59]. During this study it was not possible to directly determine the stiffness of the substrates, since the available methods (namely rheometry) required that the substrates were detached from the casting plates where they were produced, which in this case was not possible without compromising its elastic properties due to the very low stiffness of the material. Additionally, PDMS hydrogels were covalently conjugated with extracellular matrix proteins, namely Fibronectin (FN) and collagen type-I, that allowed cell adhesion to the substrates, which otherwise would not occur.

Regarding the isolation of WJ-MSCs, umbilical cord fragments exhibited the first cells migrating after around 10 days in TCPS, while on PDMS (40:1) the first cells only started migrating after 15 days [Figure

7. (A), (C),(E)]. The isolation of MSCs on PDMS substrates was typically more difficult to achieve than on TCPS, which could be related with the described lower migration and proliferation capacity that cells demonstrate when cultured on soft substrates[34,42]. Nevertheless, to our best knowledge, the isolation of WJ-MSCs was never reported using soft substrates as described here. For reasons related to experimental reproducibility and to ensure that the original cell population to be tested using distinct experimental conditions (i.e.: MSCs cultured on PDMS or TCPS) were homogeneous, the cells used for all the studies presented here were isolated on TCPS and then cultured on PDMS or TCPS from passage 2 (P2) onwards.

A previous immunophenotypic characterization revealed that the populations of cells isolated from 3 independent human umbilical cords could be considered MSCs and, according to Internal Society for Cellular Therapy[22,23] these cells also shared all minimal criteria used to define MSCs.

A kinetics proliferation study (Figure 9) was performed in order to elucidate about the proliferation potential of MSCs. Several parameters were evaluated for both substrates (TCPS or 40:1 PDMS): the total number of cells (Figure 9.D) — i.e., total number of cells that could be obtained if no cells were discarded during the analyzed passages — (TNC), population doubling (PD) rate (Figure 9.A) between each passage, and generation time (GT) (Figure 9.C) that represented the average time between two cell doublings. The TNC obtained from passage 2 (P2) to passage 6 (26 days) on TCPS ($1.61 \times 10^{10} \pm 1.12 \times 10^{10}$) was higher when compared with PDMS ($1.37 \times 10^{9} \pm 6.44 \times 10^{8}$), as well as cumulative population doubling (CPD) (Figure 9.B), which indicates how many times the population doubled (9.81 ± 1.58 for TCPS and 6.68 ±1.48 for PDMS). Despite this lower proliferative profile of MSCs cultured on 40:1 PDMS substrates when compared to TCPS, there were no statistically significant differences, except for the total number of cells in P2. Taken together, these results are somehow in agreement with previous studies showing that mammalian cell proliferation increased on substrates with higher rigidity, where the development of force-driven focal adhesions was enhanced, which lead to higher levels of FAK (focal adhesion kinase) phosphorylation, which in turn activate signaling pathways known to promote cell proliferation[28].

After cell culture conditions were well defined on both substrates (40:1 PDMS and TCPS), two different approaches were used in order to study MSCs secretome. A direct evaluation of cytokine secretion was performed using multiplex-cytokine analysis and only secretion of IL-6 and TNF-α revealed significant when cells were cultured on soft substrates (40:1 PDMS).

An unbiased analysis of the secretome and the subcellular fractions were performed by Mass Spectrometry (MS) in order to obtain the secretory and subproteomic profiles of MSCs cultured on substrates with distinct mechanical and biochemical properties.

After protein identification by Mass Spectrometry analysis, a gene ontology analysis of cellular components was performed to understand the protein profile obtained in each fraction (Secretome, Membrane and Soluble). For that, the proteins identified on the aforementioned fractions from cells cultured on TCPS or 40:1 PDMS were combined and then compared with the total number of proteins found in the study. Regarding the cellular component analysis, the results showed that there was an enrichment of proteins bellonging to cytosolic and membrane fractions of the cell, in their respective fractions and revealed that the majority of proteins found in the secretome belonged to the extracellular region. After verifying the fraction enrichment by gene ontology, a step of identification of proteins was performed. The total number of identified proteins in each cellular fraction was found and compared within both substrates under analysis (TCPS and 40:1 PDMS) in order to clarify whether the proteins were unique or shared by cells cultured on the distinct substrates. There were 896 proteins identified in MSCs secretome, 796 proteins in the soluble fraction and 1125 in the membrane fraction. The majority of the proteins found in each of the fractions was shared by both substrates and only in soluble fraction were identified more unique proteins for PDMS than for TCPS. Since the objective was to study the influence of substrate composition on the protein profile of secretome, soluble and membrane fractions, proteins were quantified to identify consequent alterations. SWATH analysis revealed a variation on secreted proteins by cells cultured on 40:1 PDMS substrates relative to those cultured on TCPS. In a total of 635 proteins, 79 showed statistically significant differences. The same was verified for the cellular subproteomes: the expression of 60 proteins varied in the soluble fraction and 62 varied in the membrane fraction. In general, all three fractions presented a higher number of down-regulated proteins than up-regulated proteins.

Within all the up-regulated proteins, the one that showed the highest value of fold change was Superoxide Dismutase [Mn] (mitochondrial), which presented an up-regulation of approximately 9 fold in the secretome. From this point, the presence of other antioxidant proteins was evaluated in all the fractions, since there was previous evidence that stem and progenitor cells can protect other cells from damaging oxygen free radicals through the production of antioxidants and anti-apoptotic molecules[60]. A total of 6 proteins with antioxidant activity were found: Peroxiredoxin-1 in the soluble fraction; Peroxiredoxin-5 (mitochondrial) and Microsomal glutathione S-transferase 3 in the membrane fraction;

Thioredoxin reductase 1 (cytoplasmic), Thioredoxin-dependent peroxide reductase (mitochondrial) and Superoxide dismutase [Mn] (mitochondrial) in the secretome.

The therapeutic potential of the secretome of mesenchymal stem cell has been reported with various types of disease models. Recently, the therapeutic effect of UC-MSCs secretome showed to play a protective role in muscle atrophied cells by blocking the oxidative stress generated by the increased reactive oxygen species (ROS). Additionally, MSCs secretome demonstrated to protect cells from apoptosis, which occurs following ROS generation in the mechanism of muscle atrophy. According to that, when muscle atrophied cells were in contact with MSCs secretome, a downregulation of oxidative stress and upregulation of antioxidant enzymes that may be beneficial to the protection of muscle mass occurred[61].

Despite the advantages of MSCs that make them suitable for cell therapy purposes, their therapeutic application has been limited due to their susceptibility to several stresses to which they are exposed during their preparation and following transplantation. Occasionally, the hostile environment that transplanted cells encounter in the host organism does not allow cell survival[62]. We propose tha MSCs cultured on soft substrates may constitute a population of cells with increased antioxidant properties, in principle allowing the cells to cope better with these stressful and hostile environments that they may encouter *in vivo*.

According to the literature, MSCs secretome includes cytokines, chemokines and growth factors and has gained increasing attention regarding its multiple implications for the repair, restoration or regeneration of injured tissues. Hence, it has been proposed that the injection of secreted molecules could be useful, rather than implanting the cells, for example for brain repair[63].

This work provides an important insight into how distinct culture substrates and conditions might be useful to modulate the phenotype of MSCs, turning these cells more suitable for specific tailor-made clinical or biotechnological purposes. It provides a large body of data that, for the purpose of the current thesis, was only detailed for a few illustrative proteins identified, in this case related with the already known important antioxidant activity of MSCs.

CHAPTER 5

CONCLUSION

5. CONCLUSION

With the present work it was possible to develop a soft substrate (40:1 PDMS) for the isolation and culture of MSCs without significant differences on proliferation kinetics in comparison with TCPS. On the other hand, this substrate seems to modulate MSCs protein profile at the level of secretome, soluble (cytosolic) and membrane fractions. This insight is based on a variation on protein levels that demonstrated the up-regulation of several proteins identified in this study. Some of these proteins are involved in metabolic processes and cellular responses related with antioxidant activity of MSCs. This work suggests that mechanotransduction may have a role in the modulation of mesenchymal stem cells' secretome and other subproteomes of the cell (namely cytosolic- and membrane-related) and according to our data may enhance the antioxidant activity of MSCs.

CHAPTER 6

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6. REFERENCES

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CHAPTER 7

SUPPLEMENTARY DATA

Table 1. SWATH-MS setup.

	m/z range	Width (Da)
Window 1	349.5-365.8	16.3
Window 2	364.8-379.3	14.5
Window 3	378.3-390.5	12.2
Window 4	389.5-400.4	10.9
Window 5	399.4-409.4	10
Window 6	408.4-418.4	10
Window 7	417.4-427	9.6
Window 8	426-435.5	9.5
Window 9	434.5-444.1	9.6
Window 10	443.1-452.2	9.1
Window 11	451.2-459.8	8.6
Window 12	458.8-467.5	8.7
Window 13	466.5-475.1	8.6
Window 14	474.1-482.3	8.2
Window 15	481.3-490	8.7
Window 16	489-497.2	8.2
Window 17	496.2-503.9	7.7
Window 18	502.9-511.1	8.2
Window 19	510.1-518.3	8.2
Window 20	517.3-525.5	8.2
Window 21	524.5-532.7	8.2
Window 22	531.7-539.9	8.2
Window 23	538.9-547.6	8.7
Window 24	546.6-554.8	8.2
Window 25	553.8-562	8.2
Window 26	561-569.2	8.2
Window 27	568.2-576.9	8.7
Window 28	575.9-584.1	8.2
Window 29	583.1-592.2	9.1
Window 30	591.2-600.3	9.1
Window 31	599.3-607.9	8.6
Window 32	606.9-616	9.1
Window 33	615-624.6	9.6
Window 34	623.6-632.7	9.1
Window 35	631.7-640.8	9.1
Window 36	639.8-649.3	9.5
Window 37	648.3-658.3	10
Window 38	657.3-667.3	10
Window 39	666.3-676.8	10.5

Window 40	675.8-686.7	10.9
Window 41	685.7-697.5	11.8
Window 42	696.5-709.2	12.7
Window 43	708.2-720.9	12.7
Window 44	719.9-732.1	12.2
Window 45	731.1-743.4	12.3
Window 46	742.4-755.5	13.1
Window 47	754.5-767.7	13.2
Window 48	766.7-780.3	13.6
Window 49	779.3-793.8	14.5
Window 50	792.8-808.2	15.4
Window 51	807.2-823.9	16.7
Window 52	822.9-841.9	19
Window 53	840.9-863.1	22.2
Window 54	862.1-885.6	23.5
Window 55	884.6-910.8	26.2
Window 56	909.8-942.7	32.9
Window 57	941.7-985	43.3
Window 58	984-1036.3	52.3
Window 59	1035.3-1109.7	74.4
Window 60	1108.7-1249.6	140.9

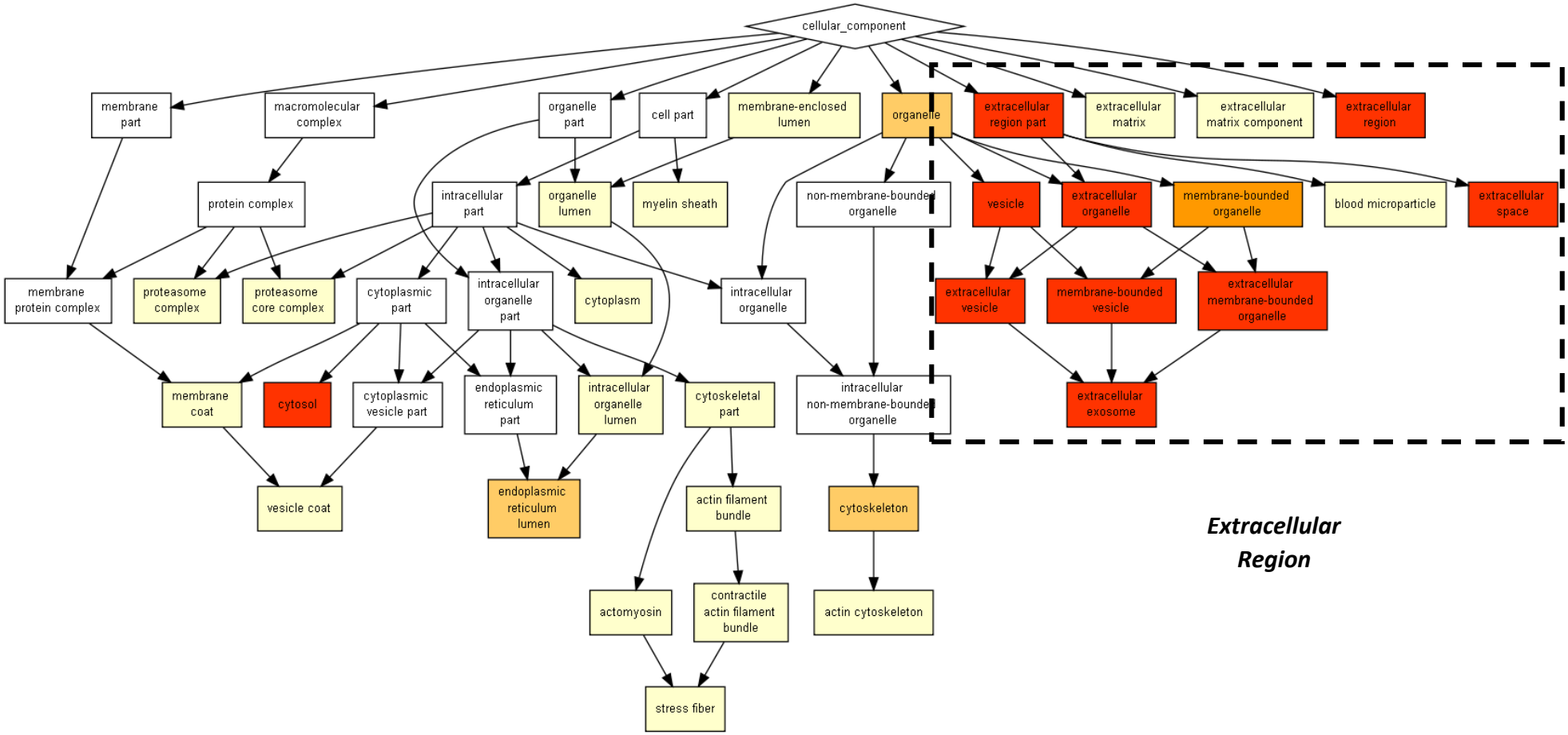

Figure S1. **Diagram representing the Gene Ontology (GO) Enrichment analysis at the cellular component level of the proteins identified in the in Secretome.** The proteins belonging to the extracellular region part were the ones with higher enrichment, demonstrated by a p-value<10^{-9}, using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – Gorilla.

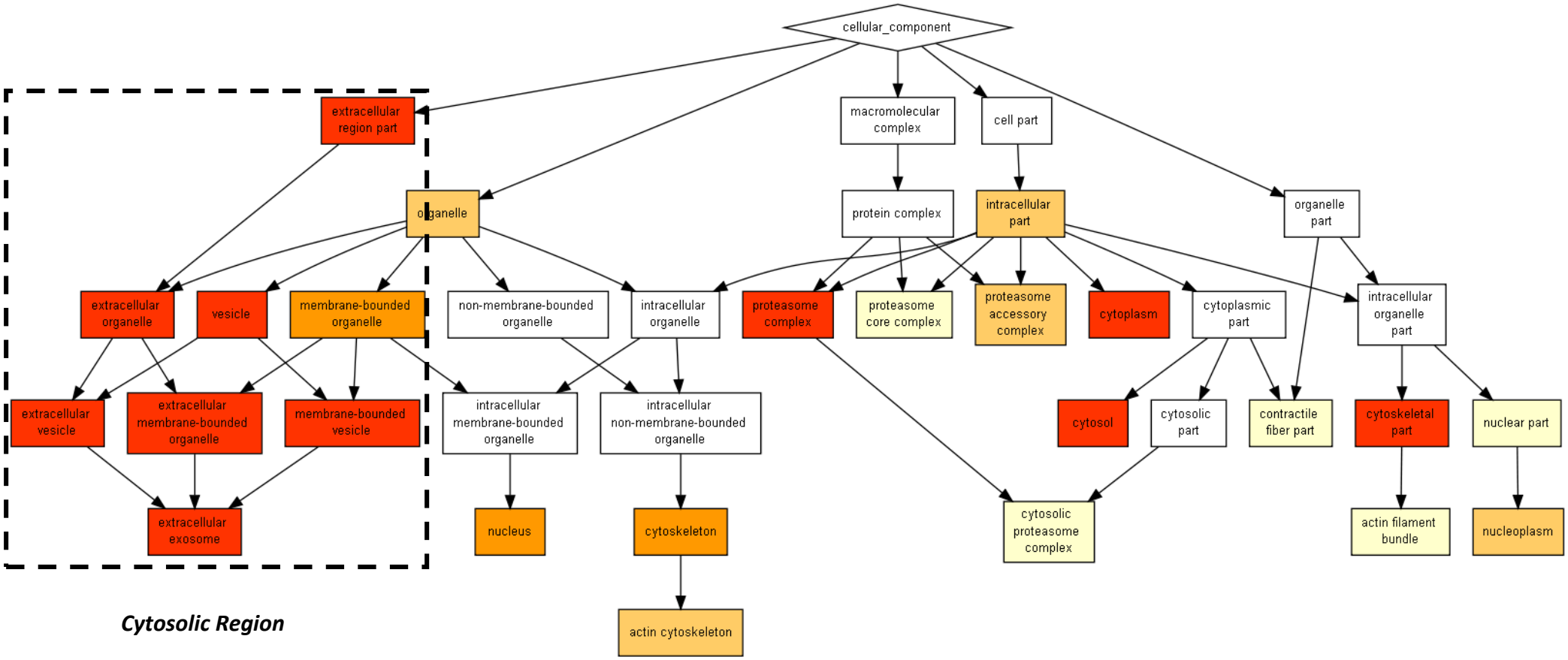

Figure S2. Diagram representing the Gene Ontology (GO) Enrichment analysis at the cellular component level of the proteins identified in the Cytosolic fraction. The proteins belonging to the extracellular region part were the ones with higher enrichment, demonstrated by a p-value<10-9, using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – Gorilla

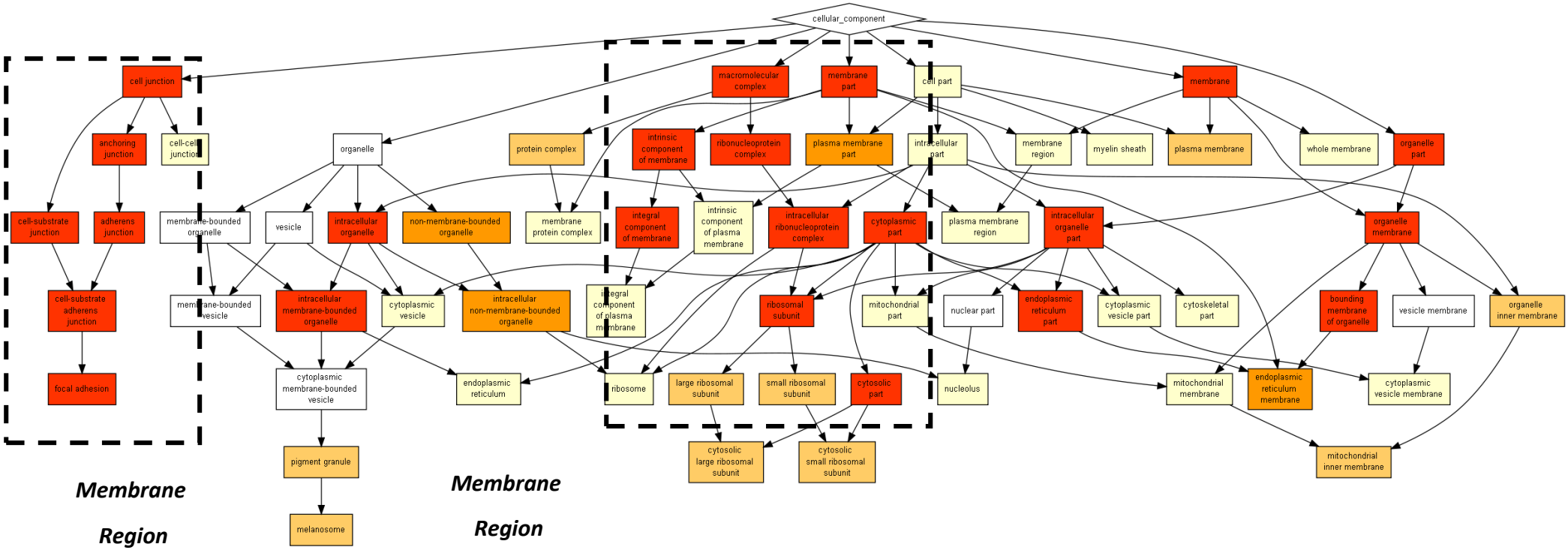

Figure S3. Diagram representing the Gene Ontology (GO) Enrichment analysis at the cellular component level of the proteins identified in the membrane fraction. The proteins belonging to the membrane region were the ones with higher enrichment, demonstrated by a p-value<10-9, using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool –Gorilla